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<p>(54) Title: STRESS PROTEIN-PEPTIDE COMPLEXES AS PROPHYLACTIC AND THERAPEUTIC VACCINES AGAINST INTRA- CELLULAR PATHOGENS (57) Abstract Disclosed is a family of vaccines that contain stress protein-peptide complexes which when administered to a mammal are operative to initiate in the mammal a cytotoxic T cell response against cells infected with a preselected intracellular pathogen. Also disclosed are methodologies for preparing and administering vaccines containing such stress protein-peptide complexes.</p>		

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STRESS PROTEIN-PEPTIDE COMPLEXES AS PROPHYLACTIC AND
THERAPEUTIC VACCINES AGAINST INTRACELLULAR PATHOGENS

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Field of the Invention

The invention relates generally to the field of vaccine development. More particularly, the invention relates to the development of prophylactic and
10 therapeutic vaccines effective against intracellular pathogens.

Background of the Invention

The development of vaccines directed against intracellular pathogens, for
15 example, viruses, bacteria, protozoa, fungi, and intracellular parasites, is ongoing. The development and use of vaccines has proved invaluable in preventing the spread of disease in man. For example, in 1967, smallpox was endemic in 33 countries with 10 to 15 million cases being reported annually. At that time, the World Health Organization introduced a program to eradicate smallpox.
20 Approximately one decade later, smallpox was successfully eradicated from the human population.

Theoretically, an ideal vaccine has a long shelf life, is capable of inducing with a single dose long lasting immunity against a preselected pathogen and all of
25 its phenotypic variants, is incapable of causing the disease to which the vaccine is directed against, is effective therapeutically and prophylactically, is prepared easily and economically using standard methodologies, and can be administered easily in the field.

- 2 -

Presently four major classes of vaccine have been developed against mammalian diseases. These include: live-attenuated vaccines; non living whole vaccines; vector vaccines; and subunit vaccines. Several reviews discuss the preparation and utility of these classes of vaccines. See for example, Subbarao et al. 5 (1992) in Genetically Engineered Vaccines, edited by Ciardi et al., Plenum Press, New York; and Melnick (1985) in High Technology Route to Virus Vaccines, edited by Dreesman et al., published by the American Society for Microbiology, the disclosures of which are incorporated herein by reference. A summary of the advantages and disadvantages of each of the four classes of vaccines is set forth 10 below.

Live attenuated vaccines comprise live but attenuated pathogens, i.e., non-virulent pathogens, that have been "crippled" by means of genetic mutations. The mutations prevent the pathogens from causing disease in the recipient or vaccinee. 15 The primary advantage of this type of vaccine is that the attenuated organism stimulates the immune system of the recipient in the same manner as the wild type pathogen by mimicking the natural infection. Furthermore, the attenuated pathogens replicate in the vaccinee thereby presenting a continuous supply of antigenic determinants to the recipient's immune system. As a result, live vaccines 20 can induce strong, long lasting immune responses against the wild type pathogen. In addition, live vaccines can stimulate the production of antibodies which neutralize the pathogen. Also they can induce resistance to the pathogen at its natural portal of entry into the host. To date, live attenuated vaccines have been developed against: smallpox; yellow fever; measles; mumps; rubella; poliomyelitis; 25 adenovirus; and tuberculosis.

Live attenuated vaccines, however, have several inherent problems. First, there is always a risk that the attenuated pathogen may revert back to a virulent phenotype. In the event of phenotypic reversion, the vaccine may actually induce

- 3 -

the disease it was designed to provide immunity against. Second, it is expensive and can be impractical to develop live vaccines directed against pathogens that continuously change their antigenic determinants. For example, researchers have been unable to develop a practical live vaccine against the influenza virus because
5 the virus continually changes the antigenic determinants of its coat proteins. Third, live attenuated vaccines may not be developed against infections caused by retroviruses and transforming viruses. The nucleic acids from these viruses may integrate into the recipients genome with the potential risk of inducing cancer in the recipient. Fourth, during the manufacture of live attenuated vaccines
10 adventitious agents present in the cells in which the vaccine is manufactured may be copurified along with the attenuated pathogen. Alien viruses that have been detected in vaccine preparations to date include the avian leukosis virus, the simian papovavirus SV40, and the simian cytomegalovirus. Fifth, live vaccine preparations can be unstable therefore limiting their storage and use in the field.
15 Presently, attempts are being made to develop stabilizing agents which enhance the longevity of the active vaccines.

Non living whole vaccines comprise non viable whole organisms. The pathogens are routinely inactivated either by chemical treatment, i.e., formalin
20 inactivation, or by treatment with lethal doses of radiation. Non living whole vaccines have been developed against: pertussis; typhus; typhoid fever; paratyphoid fever; and particular strains of influenza.

In principle, non living vaccines usually are safe to administer because it is
25 unlikely that the organisms will cause disease in the host. Furthermore, since the organism is dead the vaccines tend to be stable and have long shelf lives. There are, however, several disadvantages associated with non living whole vaccines. First, considerable care is required in their manufacture to ensure that no live pathogens remain in the vaccine. Second, vaccines of this type generally are

- 4 -

ineffective at stimulating cellular responses and tend to be ineffective against intracellular pathogens. Third, the immunity elicited by non viable vaccines is usually short-lived and must be boosted at a later date. This process repeatedly entails reaching the persons in need of vaccination and also raises the concern
5 about hypersensitizing the vaccinee against the wild type pathogen.

Vector vaccines, also known as live recombinant vehicle vaccines, may be prepared by incorporating a gene encoding a specific antigenic determinant of interest into a living but harmless virus or bacterium. The harmless vector
10 organism is in turn to be injected into the intended recipient. In theory, the recombinant vector organism replicates in the host producing and presenting the antigenic determinant to the host's immune system. It is contemplated that this type of vaccine will be more effective than the non-replicative type of vaccine. For such a vaccine to be successful, the vector must be viable, and be either naturally
15 non-virulent or have an attenuated phenotype.

Currently preferred vectors include specific strains of: vaccinia (cowpox) virus, adenovirus, adeno-associated virus, salmonella and mycobacteria. Live strains of vaccinia virus and mycobacteria have been administered safely to
20 humans in the form of smallpox and tuberculosis (BCG) vaccines, respectively. They have been shown to express foreign proteins and exhibit little or no conversion into virulent phenotypes. Several types of vector vaccines using the BCG vector currently are being developed against the human immunodeficiency virus (HIV). For example, the HIV antigenic proteins: gag; env; HIV protease;
25 reverse transcriptase; gp120 and gp41 have been introduced, one at a time, into the BCG vector and shown to induce T cell mediated immune responses against the HIV proteins in animal models (Aldovini et al. (1991) Nature 351:479-482; Stover et al. (1991) Nature 351:456-460; Colston (1991) Nature 351:442-443).

- 5 -

Vector vaccines are capable of carrying a plurality of foreign genes thereby permitting simultaneous vaccination against a variety of preselected antigenic determinants. For example, researchers have engineered several HIV genes into the vaccinia virus genome thereby creating multivalent vaccines which therefore
5 are, in theory, capable of simultaneously stimulating a response against several HIV proteins.

There are several disadvantages associated with vector vaccines. First, it is necessary to identify suitable strains of viable but non-pathogenic organisms that
10 may act as carriers for the genes of interest. Second, vector vaccines can be prepared only when a potentially protective antigenic determinants has been identified and characterized. Accordingly, vector vaccines cannot be prepared against pathogens whose antigenic determinant has not yet been identified or are so variable that the prospect of identifying the antigenic determinant for each
15 variant is impractical. Third, the genes encoding the preselected antigenic determinant must be stably transfected and expressed in the preferred carrier organism. Consequently, the methodologies required for developing this type of vaccine are both labor intensive and time consuming. Fourth, it has not yet been established that recombinant vector vaccines effectively immunize a recipient
20 against a preselected pathogen.

Subunit vaccines usually comprise a subcellular component purified from the pathogen of interest. Subunit vaccines usually are safe to administer because it is unlikely that the subcellular components will cause disease in the recipient. The
25 purified subcellular component may be either a defined subcellular fraction, purified protein, nucleic acid or polysaccharide having an antigenic determinant capable of stimulating an immune response against the pathogen. The antigenic components can be purified from a preparation of disrupted pathogen. Alternatively, the antigenic proteins, nucleic acids or polysaccharides may be

- 6 -

synthesized using procedures well known in the art. Diseases that have been treated with subunit type vaccines include: cholera; diphtheria; hepatitis type B; poliomyelitis; tetanus; and specific strains of influenza.

5 There are, however, several disadvantages associated with subunit vaccines. First, it is important to identify and characterize the protective antigenic determinant. This can be a labor intensive and time consuming process. As a result it may be impractical to develop subunit vaccines against pathogens with highly variable antigenic determinants. Second, subunit vaccines generally are
10 ineffective at stimulating cytotoxic T cell responses and so they may be ineffective at stimulating an immune response against intracellular pathogens. Third, the immunity elicited by subunit vaccines is usually short-lived, and like the non living whole vaccines must be boosted at a later date therefore raising the concern about hypersensitizing the vaccinee against the wild type pathogen.

15 Heretofore, many of the inactivated whole and subunit vaccines have not been sufficiently immunogenic by themselves to induce strong, protective responses. As a result, immunostimulants including, for example, aluminum hydroxide; intact mycobacteria; and/or mycobacterial components have been co-
20 administered with these vaccines to enhance the immune response stimulated by the vaccine. Recently, experiments have shown that mycobacterial heat shock proteins may act as carriers for peptide vaccines thereby enhancing the immunogenicity of the peptides in vivo (Lussow et al. (1991) Eur. J. Immunol. 21:2297-2302). Further studies have shown that administering a composition to
25 mice comprising an antigenic peptide chemically crosslinked to a purified mycobacterial stress protein stimulates a humoral (antibody mediated) rather than a temporal (cell mediated) response against the antigenic peptide (Barrios et al. (1992) Eur. J. Immunol. 22:1365-1372).

- 7 -

However, because it is generally believed that cellular responses are required for immunizing against intracellular pathogens (see for example, "Advanced Immunology," Male *et al.* (1991) Gower Medical Publishing; Raychaudhuri *et al.* (1993) *Immunology Today* 14: 344-348) it is contemplated that
5 conventional subunit and inactivated whole organism vaccines may be ineffective at stimulating immune responses, specifically cytotoxic T cell responses, against intracellular pathogens.

It is an object of the instant invention to provide a safe subunit vaccine
10 comprising a stress protein-peptide complex for administration to a mammal that is capable of inducing, by means of a cytotoxic T cell response, resistance to infection by a preselected intracellular pathogen. The vaccines prepared in accordance with the invention may be used to elicit an immune response against an intracellular
15 pathogens whose antigenic determinants have been identified, have not yet been identified, or where it is impractical to isolate and characterize each of the antigenic determinants. The vaccines prepared in accordance with the invention may be prophylactically and therapeutically effective against preselected pathogens.

Another object of the invention is to provide a method for inducing in a
20 mammal resistance to infection by an intracellular pathogen by administering to the mammal a stress protein-peptide subunit vaccine. Another object is to provide a method for rapidly and cost effectively producing commercially feasible quantities of the stress protein-peptide vaccines from a cell or cell line infected with the intracellular pathogen or alternatively from a cell or cell line transfected with,
25 and expressing a gene encoding a specific antigenic determinant. Still another object is to provide a method for preparing an immunogenic stress protein-peptide subunit vaccine by reconstituting *in vitro* immunologically unreactive stress proteins and peptides thereby to produce immunoreactive complexes capable of stimulating an immune response against a preselected intracellular pathogen.

- 8 -

These and other objects and features of the invention will be apparent from the description, drawings, and claims which follow.

- 9 -

Summary of the Invention

5 It has now been discovered that a subunit vaccine containing a stress protein-peptide complex when isolated from cells infected with a preselected intracellular pathogen and then administered to a mammal can effectively stimulate cellular immune responses against cells infected with the same pathogen. Specifically, the immune response is mediated through the cytotoxic T cell cascade which targets and destroys cells containing intracellular pathogens.

10

The vaccines prepared in accordance with the methodologies described herein provide an alternative approach for stimulating cellular immunity thereby obviating the use of live (attenuated or otherwise) intracellular pathogens. In addition, the vaccines described herein are ideal for inducing immune responses against intracellular pathogens having either defined or as yet undefined immunogenic determinants. Furthermore the vaccines may be used to induce immune responses against intracellular pathogens whose antigenic determinants are either diverse or constantly changing thereby making the isolation and characterization of antigenic determinants impractical.

15
20

In a preferred aspect, the invention comprises a vaccine that can be administered to a mammal for inducing in the mammal a cytotoxic T cell response against a preselected intracellular pathogen. Also, it is contemplated that the vaccines may induce in the mammal, by means of a cytotoxic T cell response, resistance to infection by the preselected intracellular pathogen. The vaccines manufactured in accordance with the principles described herein contain an immunogenic stress protein-peptide complex that is capable of stimulating in the recipient a cytotoxic T cell response directed against cells infected with the pathogen of interest. The complex when combined with a pharmaceutically

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- 10 -

acceptable carrier, adjuvant, or excipient may be administered to a mammal using techniques well known in the art.

The term "vaccine", as used herein, is understood to mean any composition
5 containing a stress protein-peptide complex having at least one antigenic
determinant which when administered to a mammal stimulates in the mammal an
immune response against the antigenic determinant.

The term "stress protein" as used herein, is understood to mean any cellular
10 protein which satisfies the following criteria. It is a protein whose intracellular
concentration increases when a cell is exposed to stressful stimuli, is capable of
binding other proteins or peptides, and is capable of releasing the bound proteins
or peptides in the presence of adenosine triphosphate (ATP) or low pH. Stressful
stimuli include, but are not limited to, heat shock, nutrient deprivation, metabolic
15 disruption, oxygen radicals, and infection with intracellular pathogens.

It will be apparent to the artisan upon reading this disclosure that other
recombinant stress proteins, including non native forms, truncated analogs,
muteins, fusion proteins as well as other proteins capable of mimicking the peptide
20 binding and immunogenic properties of a stress protein may be used in the
preparation of stress protein-peptide vaccines disclosed herein.

The first stress proteins to be identified were the heat shock proteins (Hsp).
As their name suggests, Hsps are induced by a cell in response to heat shock.
25 Three major families of Hsp have been identified and are called Hsp60, Hsp70 and
Hsp90 because of their respective molecular weights of about 60, 70, and 90 kD.
Many members of these families subsequently were found to be induced in
response to other stressful stimuli, such as those mentioned above.

- 11 -

Stress proteins are found in all prokaryotes and eukaryotes and exhibit a remarkable level of evolutionary conservation. For example, DnaK, the Hsp70 from *E. coli* has about 50% amino acid sequence identity with Hsp70 proteins from eukaryotes (Bardwell *et al.* (1984) *Proc. Natl. Acad. Sci.* 81:848-852). The Hsp60 and Hsp90 families also exhibit similarly high levels of intrafamilial conservation (Hickey *et al.* (1989) *Mol. Cell Biol.* 9:2615-2626; Jindal (1989) *Mol. Cell Biol.* 9:2279-2283). In addition, it has been discovered that the Hsp-60, Hsp-70, and Hsp-90 families are composed of proteins that are related to the stress proteins in sequence, for example, having greater than 35% amino acid identity, but whose expression levels typically remain unaltered under conditions stressful to the host cell. An example of such a protein includes the constitutively expressed cytosolic protein Hsc 70 to which is related in amino acid sequence to the stress-induced protein Hsp 70. Accordingly, it is contemplated the definition of stress protein, as used herein, embraces other proteins, muteins, analogs, and variants thereof having at least 35% to 55%, preferably 55% to 75%, and most preferably 75% to 95% amino acid identity with members of the three families whose expression levels in a cell are stimulated in response to stressful stimuli.

The term "peptide", as used herein, is understood to mean any amino acid sequence that is present in a eukaryotic cell infected with an intracellular pathogen but which is not present in a similar cell when the cell is not infected with the same pathogen. The definition embraces peptides that not only originate from the pathogen itself but also peptides which are synthesized by the infected cell in response to infection by the intracellular pathogen.

The term "immunogenic stress protein-peptide complex", as used herein, is understood to mean any complex containing a stress protein and a peptide that is capable of inducing an immune response in a mammal. The peptides preferably are non covalently associated with the stress protein. The complexes may include,

- 12 -

but are not limited to, Hsp60-peptide, Hsp70-peptide and Hsp90-peptide complexes. In a preferred aspect of the invention a stress protein belonging to the Hsp90 family, namely gp96 can be used to generate an effective vaccine containing a gp96-peptide complex. Since the peptides can be dissociated from the complex in
5 the presence of ATP or low pH potentially antigenic peptides can be isolated from cells infected with a preselected intracellular pathogen. Consequently, the antigenic determinants for potentially any intracellular pathogen of interest can be identified readily using the methodologies described herein.

10 The term "cytotoxic T cell", as used herein, is understood to mean any T lymphocyte expressing the cell surface glycoprotein marker CD8 that is capable of targeting and lysing a target cell which bears a class I histocompatibility complex on its cell surface and which is infected with an intracellular pathogen. The term "cytotoxic T cell response" is understood to mean any cytotoxic activity that is
15 mediated by cytotoxic T cells.

As used herein, the term "intracellular pathogen" is understood to mean any viable organism, including, but not limited to, viruses, bacteria, fungi, protozoa and intracellular parasites, capable of existing within a mammalian cell and causing a
20 disease in the mammal.

In a preferred aspect of the invention, the stress protein-peptide vaccines have particular utility in treating human diseases caused by intracellular pathogens. It is contemplated that the vaccines developed using the principles
25 described herein will be useful in treating diseases of other mammals, for example, farm animals including: cattle; horses; goats; sheep; and pigs, and household pets including: cats; and dogs.

- 13 -

Vaccines may be prepared that stimulate cytotoxic T cell responses against cells infected with viruses including, but not limited to, hepatitis type A, hepatitis type B, hepatitis type C, influenza, varicella, adenovirus, herpes simplex type I (HSV-I), herpes simplex type II (HSV-II), rinderpest, rhinovirus, echovirus, 5 rotavirus, respiratory syncytial virus, papilloma virus, papova virus, cytomegalovirus, echinovirus, arbovirus, huntavirus, coxsachie virus, mumps virus, measles virus, rubella virus, polio virus, human immunodeficiency virus type I (HIV-I), and human immunodeficiency virus type II (HIV-II). Vaccines also may be prepared that stimulate cytotoxic T cell responses against cells infected with 10 intracellular bacteria, including, but not limited to, *Mycobacteria*, *Rickettsia*, *Mycoplasma*, *Neisseria* and *Legionella*. Vaccines also may be prepared that stimulate cytotoxic T cell responses against cells infected with intracellular protozoa, including, but not limited to, *Leishmania*, *Kokzidioa*, and *Trypanosoma*. Vaccines may be prepared that stimulate cytotoxic T cell responses against cells infected with 15 intracellular parasites including, but not limited to, *Chlamydia* and *Rickettsia*.

In another preferred embodiment of the invention, the stress protein-peptide vaccine may also contain a therapeutically effective amount of a cytokine. As used herein, the term "cytokine" is meant to mean any secreted polypeptide that 20 influences the function of other cells mediating an immune response. Currently, preferred cytokines include: interleukin-1 α (IL-1 α), interleukin-1 β (IL-1 β), interleukin-2 (IL-2), interleukin-3 (IL-3), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-7 (IL-7), interleukin-8 (IL-8), interleukin-9 (IL-9), interleukin-10 (IL-10), interleukin-11 (IL-11), interleukin-12 (IL-12), interferon α 25 (IFN α), interferon β (IFN β), interferon γ (IFN γ), tumor necrosis factor α (TNF α), tumor necrosis factor β (TNF β), granulocyte colony stimulating factor (G-CSF), granulocyte/macrophage colony stimulating factor (GM-CSF), and transforming growth factor β (TGF- β). It is contemplated that other but as yet undiscovered cytokines may be effective in the invention. In addition, conventional antibiotics

- 14 -

may be co-administered with the stress protein-peptide complex. The choice of a suitable antibiotic or a combination thereof, however, will be dependent upon the disease in question.

5 It has been discovered that the vaccine stimulates the cytotoxic T cell response via the major histocompatibility complex (MHC) class I cascade. Thus, it is contemplated that the cytotoxic T cell response may be enhanced further by co-administering the vaccine with a therapeutically effective amount of one or more of cytokines that potentiate or modulate cytotoxic T cell responses.

10

 Another preferred embodiment, the invention provides a method for stimulating in a mammal a cellular immune response, specifically a cytotoxic T cell response, against cells infected with a preselected intracellular pathogen. The method involves administering to the mammal a vaccine made in accordance with
15 the principles disclosed herein in an amount sufficient to elicit in the mammal a cytotoxic T cell response against the preselected intracellular pathogen.

 The vaccine may be administered prophylactically to a mammal in order to stimulate in the mammal a cytotoxic T cell response that prevents subsequent
20 infection of the mammal by the intracellular pathogen. Alternatively, the vaccine may be administered therapeutically to a mammal having a disease caused by an intracellular pathogen. It is contemplated that the vaccine may stimulate a cytotoxic T cell response against cells presently infected with the intracellular pathogen.

25

 The dosage and means of administration of the family of stress protein-peptide vaccines necessarily will depend upon the nature of the complex, the intracellular pathogen and the nature of the disease in question. The complex should be administered in an amount sufficient to initiate a cytotoxic T cell

- 15 -

response against the intracellular pathogen. In general, the amount of stress protein-peptide complex administered may range from about 0.1 to about 1000 micrograms of complex/kg body weight of the mammal/immunization, and preferably in the range of about 0.5 to 100 micrograms of complex/kg body weight of the mammal/immunization. The recipient preferably should be vaccinated four times at weekly intervals. If necessary, the responses may be boosted at a later date by subsequent administration of the vaccine. It is contemplated, however, that the optimal dosage and vaccination schedule may be determined empirically for each stress protein-peptide vaccine complex by an artisan using conventional techniques well known in the art.

In another aspect, the invention provides a variety of methodologies for preparing commercially available amounts of the stress-protein peptide vaccines which when administered to a mammal induce in the mammal a cytotoxic T cell response against cells infected with a preselected antigen. In one approach, the stress protein-peptide complex may be harvested using conventional protein purification methodologies from a sample of tissue, an isolated cell or immortalized cell line infected with the preselected intracellular pathogen, or an isolated cell or immortalized cell line transfected with, and expressing a gene encoding a preselected antigenic determinant. The purified complex subsequently may be stored or combined with a pharmaceutically acceptable carrier for administration as a vaccine.

Alternatively, the stress protein-peptide complex may be prepared by reconstituting a potentially antigenic peptide and a stress protein in vitro. For example, the antigenic peptide may be eluted from either a purified stress protein-peptide complex or a MHC-peptide complex using methodologies well known in the art. Specifically, the peptides may be eluted from the stress protein-peptide complex by incubating the complex in the presence of ATP or low pH.

- 16 -

Alternatively, the peptides may be eluted from the MHC-peptide complex by incubating the complex in the presence of trifluoroacetic acid (TFA). The resulting peptides may be purified by reverse phase HPLC and their amino acid sequences determined by standard protein sequencing methodologies. Peptides of defined
5 sequence then may be synthesized using conventional peptide synthesis methodologies. Stress proteins may be purified directly from cells naturally expressing the stress proteins. Alternatively, recombinant stress proteins, including non native forms, truncated analogs, muteins, fusion proteins as well as other constructs capable of mimicking the peptide binding and immunogenic
10 properties of stress proteins may be expressed using conventional recombinant DNA methodologies. For example, a recombinant stress protein may be expressed from recombinant DNA in either a eukaryotic or prokaryotic expression system and purified from the expression system. The two purified components then may be combined in vitro to generate a synthetic and completely defined stress protein-
15 peptide complex. The immunogenicity and specificity of the recombinant-complexes subsequently may be assayed in vitro and in vivo to identify useful candidate complexes that stimulate cytotoxic T cell responses against a preselected intracellular pathogen. Once identified, the synthetic complexes may be prepared on any scale, stored as is, or combined with pharmaceutically acceptable carriers for
20 administration to mammals.

- 17 -

Brief Description of the Drawings

The foregoing and other objects and features of the invention, as well as the
5 invention itself, may be more fully understood from the following description,
when read together with the accompanying drawings, in which:

Figure 1 shows antigen specific cytotoxic T cell activity of splenocytes
derived from mice immunized with a gp96-peptide complex harvested from
10 BALB/c fibroblasts transfected with the nucleoprotein (NP) gene from the PR8
influenza virus. The cytotoxic activity was assayed by the release of ^{51}Cr from
BALB/c fibroblasts expressing the NP gene (filled circles), BALB/c fibroblasts
expressing the NP gene but treated with the anti-MHC type I antisera K44 (empty
circles) and from the syngeneic non-NP transfected cell line 5117 (asterisks).
15

Figure 2 shows antigen specific cytotoxic T cell activity of splenocytes
derived from mice immunized with gp96-peptide complex harvested from SV40
transformed SVB6 cells. The cytotoxic activity was assayed by the release of ^{51}Cr
from SVB6 cells (filled circles) and from a non-SV40 transformed syngeneic cell line,
20 MCA (empty circles).

Figure 3A-3D shows antigen specific cytotoxic T cell activities of splenocytes
derived from two mice immunized with a reconstituted Hsp70-peptide complex
where the peptide has the sequence SLSDLRGYVYQGL (SEQ. ID. NO. 1). Prior to
25 performing the assay, the splenocytes derived from each mouse were stimulated
either once (3A and 3C) or twice (3B and 3D) *in vitro* with lethally irradiated cells
transfected with, and expressing the peptide SLSDLRGYVYQGL (SEQ. ID. NO. 1).
Cytotoxic activity was assayed by the release of ^{51}Cr from EL4 cells expressing the

- 18 -

peptide (filled triangle) and from EL4 cells not expressing the peptide (empty triangles).

Detailed Description.

The invention is based on the discovery that a stress protein-peptide
5 complex when isolated from a eukaryotic cell infected with a preselected
intracellular pathogen and then administered to a mammal can stimulate a
cytotoxic T cell response directed against cells infected with the same pathogen.
This discovery provides a significant advance to the field of vaccine development.

10 In accordance with the invention, the aforementioned discovery is exploited
to provide a family of vaccines which may be used to immunize mammals against
diseases caused by intracellular pathogens. In principle, the vaccines can be
prepared against any intracellular pathogen of interest, for example: viruses;
bacteria; protozoa; fungi; or intracellular parasites. Generic methodologies useful
15 for preparing vaccines against all of these classes of pathogens are discussed in
detail hereinbelow.

As will be appreciated by those skilled in the art, the stress protein-peptide
vaccines described herein have several advantages over the vaccines currently
20 available. First, the stress protein-peptide vaccines provide an alternative approach
for stimulating cellular immunity and obviate the use of intact intracellular
(attenuated or otherwise) pathogens. Second, since the vaccines do not contain
intact organisms this reduces the risk of causing the disease the vaccine was
designed to induce immunity against. Third, the vaccines described herein are
25 ideal for inducing immune responses against either defined antigenic determinants
isolated from an intracellular pathogen or as yet undefined antigenic determinants.
Furthermore, vaccines may be prepared that are effective against pathogens that
normally evade the immune system by evolving new antigenic coat proteins, i.e.,
the influenza virus. Fourth, vaccines of this type may in principle be prepared

- 20 -

against any intracellular pathogen of interest. Fifth, the vaccines may be prepared synthetically using the methodologies described hereinafter thereby providing completely defined vaccines that are suitable for administration to humans.

5 It is contemplated that the vaccines may be administered either prophylactically or therapeutically. When administered prophylactically the vaccine may stimulate in the mammal a cytotoxic T cell response that permits the vaccinee to resist subsequent infection by the intracellular pathogen. Alternatively, when administered therapeutically the vaccine may stimulate in the mammal a
10 cytotoxic T cell response against a pathogen which is presently infecting and causing disease in the mammal.

The specific component of the vaccine that induces in the recipient a specific cytotoxic T cell response against the pathogen is a stress protein-peptide complex.

15 The peptide may be any amino acid sequence that is present in a eukaryotic cell infected with an intracellular pathogen but which is not present when such a cell is not infected with the same pathogen. This includes peptides that not only originate from the pathogen itself but also are synthesized by the infected cell in response to infection by the intracellular pathogen.

20

The immunogenic complexes may be purified from any eukaryotic cell, including: whole tissues; isolated cells; and immortalized eukaryotic cell lines infected with the intracellular pathogen. The complexes may be purified by using conventional protein purification techniques well known in the art. For example, it
25 is contemplated that an immunogenic complex capable of stimulating a cytotoxic T cell response against the influenza virus may be harvested from a eukaryotic cell line that is infected with the influenza virus.

- 21 -

In addition, it has been found that the peptide can be eluted from the stress protein-complex either in the presence of ATP or low pH. Neither the peptide nor the stress protein individually are effective at inducing a cytotoxic T cell response. These experimental conditions, however, may be exploited to isolate peptides from
5 infected cells which may contain potentially useful antigenic determinants. Once isolated, the amino acid sequence of each antigenic peptide may be determined using conventional amino acid sequencing methodologies. Consequently, the antigenic determinants for potentially any intracellular pathogen of interest can be identified readily using the methodologies described herein. As discussed in detail
10 hereinafter, this property may be exploited in the preparation of completely synthetic vaccines.

Similarly, it has been found that potentially immunogenic peptides may be eluted from MHC-peptide complexes using techniques well known in the art. See
15 for example, Falk *et al.* (1990) Nature 348:248-251; Rotzsche *et al.* (1990) Nature 348:252-254; Elliott *et al.* (1990) Nature 348:195-197; Falk *et al.* (1991) Nature 351:290-296, Demotz *et al.* (1989) Nature 334:682-684; Rotzsche *et al.* (1990) Science 249:283-287, the disclosures of which are incorporated herein by reference. Although the peptides eluted from the MHC complexes may define a potentially
20 protective antigenic determinant, it is appreciated that administration of the isolated peptide in a conventional subunit vaccine may be ineffective at stimulating a cytotoxic T cell response in the recipient. Consequently, it is contemplated that the peptides eluted from MHC-peptide complexes may be reconstituted with a stress protein, using the methodologies described herein, thereby to generate a
25 stress protein-peptide complex effective at stimulating a cytotoxic T cell response capable of targeting and lysing cells expressing the antigenic peptide.

Stress proteins useful in the practice of the instant invention may be defined as any cellular protein that satisfies the following criteria. It is a protein whose

- 22 -

intracellular concentration increases when a cell is exposed to a stressful stimuli, it is capable of binding other proteins or peptides, and it is capable of releasing the bound proteins or peptides in the presence of adenosine triphosphate (ATP) or low pH.

5

The first stress proteins to be identified were the heat shock proteins (Hsp). As their name implies, Hsps are synthesized by a cell in response to heat shock. To date, three major families of Hsp have been identified based on molecular weight. The families have been called Hsp60, Hsp70 and Hsp90 where the numbers reflect the approximate molecular weight of the stress proteins in kD. Many members of these families subsequently were found to be induced in response to other stressful stimuli including, but not limited to, nutrient deprivation, metabolic disruption, oxygen radicals, and infection with intracellular pathogens. See for example: Welch (May 1993) Scientific American 56-64; Young (1990) Annu. Rev. Immunol. 8:401-420; Craig (1993) Science 260:1902-1903; Gething et al. (1992) Nature 355:33-45; and Lindquist et al. (1988) Annu. Rev. Genetics 22:631-677, the disclosures of which are incorporated herein by reference. Accordingly, it is contemplated that stress proteins belonging to all three families may be useful in the practice of the instant invention.

20

The major stress proteins can accumulate to very high levels in stressed cells, but they occur at low to moderate levels in cells that have not been stressed. For example, the highly inducible mammalian Hsp70 is hardly detectable at normal temperatures but becomes one of the most actively synthesized proteins in the cell upon heat shock (Welch et al. (1985), J. Cell. Biol. 101:1198-1211). In contrast, Hsp90 and Hsp60 proteins are abundant at normal temperatures in most, but not all, mammalian cells and are further induced by heat (Lai et al. (1984), Mol. Cell. Biol. 4:2802-10; van Bergen en Henegouwen et al. (1987), Genes Dev. 1:525-531).

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- 23 -

Stress proteins are among the most highly conserved proteins in existence. For example, DnaK, the Hsp70 from E. coli has about 50% amino acid sequence identity with Hsp70 proteins from eukaryotes (Bardwell et al. (1984) Proc. Natl. Acad. Sci. 81:848-852). The Hsp60 and Hsp90 families also show similarly high levels of intrafamilial conservation (Hickey et al. (1989) Mol. Cell Biol. 9:2615-2626; Jindal (1989) Mol. Cell Biol. 9:2279-2283). In addition, it has been discovered that the Hsp60, Hsp70 and Hsp90 families are composed of proteins that are related to the stress proteins in sequence, for example, having greater than 35% amino acid identity, but whose expression levels typically remain unaltered under conditions stressful to the host cell. An example of such a protein includes the constitutively expressed cytosolic protein Hsc 70 which is related in amino acid sequence to the stress-induced protein Hsp 70. It is, therefore, contemplated that the definition of stress protein, as used herein, embraces other proteins, muteins, analogs, and variants thereof having at least 35% to 55%, preferably 55% to 75%, and most preferably 75% to 95% amino acid identity with members of the three families whose expression levels in a cell are enhanced in response to a stressful stimulus. The purification of stress proteins belonging to these three families is described below.

The immunogenic stress protein-peptide complexes of the invention may include any complex containing a stress protein and a peptide that is capable of inducing an immune response in a mammal. The peptides preferably are non covalently associated with the stress protein. Preferred complexes may include, but are not limited to, Hsp60-peptide, Hsp70-peptide and Hsp90-peptide complexes. For example, a stress protein called gp96 which is present in the endoplasmic reticulum of eukaryotic cells and is related to the cytoplasmic Hsp90s can be used to generate an effective vaccine containing a gp96-peptide complex.

- 24 -

Another family of low molecular weight heat shock proteins has now been identified and is called Hsp 25/Hsp 27. The purification of these proteins is discussed below. It is contemplated that these low molecular weight proteins may also have utility in the instant invention.

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It has been discovered also that the stress protein-peptide complexes of the invention can be prepared from cells infected with an intracellular pathogen as well as cells that have been transformed by an intracellular pathogen. For example, immunogenic stress protein peptide complexes may be isolated from eukaryotic cells transformed with a transforming virus such as SV40, see below.

10

In a preferred aspect of the invention, the purified stress protein-peptide vaccines may have particular utility in the treatment of human diseases caused by intracellular pathogens. It is appreciated, however, that the vaccines developed using the principles described herein will be useful in treating diseases of other mammals, for example, farm animals including: cattle; horses; sheep; goats; and pigs, and household pets including: cats; and dogs, that similarly are caused by intracellular pathogens.

15

In accordance with the methods described herein, vaccines may be prepared that stimulate cytotoxic T cell responses against cells infected with viruses including, but not limited to, hepatitis type A, hepatitis type B, hepatitis type C, influenza, varicella, adenovirus, HSV-I, HSV-II, rinderpest rhinovirus, echovirus, rotavirus, respiratory syncytial virus, papilloma virus, papova virus, cytomegalovirus, echinovirus, arbovirus, huntavirus, coxsachie virus, mumps virus, measles virus, rubella virus, polio virus, HIV-I, and HIV-II. Similarly, vaccines may also be prepared that stimulate cytotoxic T cell responses against cells infected with intracellular bacteria, including, but not limited to, *Mycobacteria*, *Rickettsia*, *Mycoplasma*, *Neisseria* and *Legionella*. In addition, vaccines may also be

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- 25 -

prepared that stimulate cytotoxic T cell responses against cells infected with intracellular protozoa, including, but not limited to, *Leishmania*, *Kokzidioa*, and *Trypanosoma*. Furthermore, vaccines may be prepared that stimulate cytotoxic T cell responses against cells infected with intracellular parasites including, but not
5 limited to, *Chlamydia* and *Rickettsia*.

I. Propagation of infected eukaryotic cells.

As will be appreciated by those skilled in the art, the protocols described
10 herein may be used to isolate stress protein-peptide complexes from any eukaryotic cell, for example, tissues, isolated cells or immortalized eukaryotic cell lines infected with a preselected intracellular pathogen.

When immortalized animal cell lines are used as a source of the stress
15 protein-peptide complex it is of course important to use cell lines that can be infected with the pathogen of interest. In addition, it is preferable to use cells that are derived from the same species as the intended recipient of the vaccine.

For example, in order to prepare a stress protein-peptide complex for
20 administration to humans that may be effective against HIV-I, the virus may be propagated in human cells which include, but are not limited to, human CD4+ T cells, HepG2 cells, and U937 promonocytic cells. In order to prepare a stress protein-peptide complex for administration to humans that may be effective against HIV-II, the virus may be propagated in, for example, human CD4+ T cells.
25 Similarly, influenza viruses may be propagated in, for example, human fibroblast cell lines and MDCK cells, and mycobacteria may be cultured in, for example, human Schwann cells.

- 26 -

If the intracellular pathogens do not lyse the infected cells then the infected cells are cultured under the same conditions as the normal uninfected cells. For example, mycobacteria may be propagated in nerve cultures of the sensory ganglia of newborn Swiss white mice. The nerve cells are cultured in a growth medium
5 containing 70% Dulbecco modified Eagle minimal essential medium (DMEM) with 0.006% glucose, 20% fetal calf serum, 10% chicken embryo extract and cytosine arabinoside. After eight to ten days, the cultures are inoculated with $5-8 \times 10^6$ mycobacteria isolated from fresh nodules of untreated lepromatous leprosy patients. The infected cells may be cultured at 37°C, for up to 6 weeks, after which
10 the infected cells are harvested and the stress protein-peptide complexes isolated. See for example, Mukherjee *et al.* (1985) *J. Clin. Micro.* 21:808-814, the disclosure of which is incorporated herein by reference.

If, on the other hand, the host cells are lysed by the pathogen of interest (as
15 in the case of influenza virus) the cells may still be grown under standard conditions except the cells are washed and harvested just prior to lysis of the host cell. For example, during the purification of stress protein-peptide complexes from influenza infected cells, fibroblasts (or other cell types) are infected for 1 hour at 37°C with 5-10 plaque forming units (PFU) of virus per cell. The infected cells may
20 be cultured in plain DMEM medium for 24 hours at 37°C. After 24 hours the cells are washed and harvested prior to lysis. The stress protein-peptide complexes may be isolated using the procedures set forth below.

In addition, when the gene encoding a particular antigenic determinant has
25 been identified, the gene of interest may be transfected and expressed in an immortalized human or other mammalian cell line using techniques well known in the art. See for example "Current Protocols in Molecular Biology" (1989), eds. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA and Struhl K, Wiley Interscience, the disclosure of which is incorporated by reference herein. The

- 27 -

transfected cells may be grown under standard conditions and the complexes isolated subsequently.

II. Preparation of Stress Proteins and Immunogenic Stress Protein-peptide complexes.

Methods for preparing Hsp70-peptide complexes, Hsp90-peptide complexes, gp96-peptide complexes, Hsp70, Hsp25/Hsp27, and Hsp60 are set forth below.

(a) Purification of Hsp70-peptide complexes.

A pellet of infected cells is resuspended in 3 volumes of 1X Lysis buffer consisting of 5mM sodium phosphate buffer (pH7), 150mM NaCl, 2mM CaCl₂, 2mM MgCl₂ and 1mM phenyl methyl sulfonyl fluoride (PMSF). The pellet is sonicated, on ice, until >99% cells are lysed as judged by microscopic examination. Alternatively, the cells may be lysed by mechanical shearing. In this procedure, the cells are resuspended in 30mM sodium bicarbonate pH 7.5, 1mM PMSF, incubated on ice for 20 min. and then homogenized in a dounce homogenizer until >95% cells are lysed.

The lysate is centrifuged at 1000g for 10 minutes to remove unbroken cells, nuclei and other debris. The supernatant from this centrifugation step is then recentrifuged at 100,000g for 90 minutes.

The supernatant is mixed for 2-3 hours at 4°C with Con A Sepharose equilibrated with PBS containing 2mM Ca²⁺ and 2mM Mg²⁺. When the cells are lysed by mechanical shearing, the supernatant is diluted with equal volume of 2X Lysis Buffer before proceeding. Then the slurry is packed into a column and

- 28 -

washed with 1X lysis buffer. The material that does not bind is dialyzed for 36 hours (three times, 100 volumes each time) against 10mM Tris-Acetate pH 7.5, 0.1 mM EDTA, 10mM NaCl, 1mM PMSF. The dialyzate is centrifuged for 20 min. at 17,000 rpm (Sorvall SS34 rotor) and the resulting supernatant applied to a Mono Q FPLC column (Pharmacia) equilibrated in 20mM Tris-Acetate pH 7.5, 20 mM NaCl, 0.1mM EDTA and 15mM 2-mercaptoethanol. Then the proteins are eluted with a 20 mM to 500 mM NaCl gradient. The fractions are characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting using an appropriate anti-Hsp70 antibody (such as clone N27F3-4 from StressGen).

The fractions that are strongly immunoreactive with the antibody are pooled and the Hsp70-peptide complexes precipitated with ammonium sulfate. The complex is precipitated in the 50%-70% ammonium sulfate cut. The protein pellet is harvested by centrifugation at 17,000 rpm (SS34 Sorvall rotor) and washed with 70% ammonium sulfate. Then the pellet is solubilized and the residual ammonium sulfate removed by gel filtration on a Sephadex® G25 column (Pharmacia).

The Hsp70-peptide complex can be purified to apparent homogeneity using this method. Up to 1mg of Hsp70-peptide complex can be purified from 1g of cells/tissue.

(b) Purification of Hsp70.

25

The Hsp70 polypeptide may be purified from the Hsp70-peptide complex by ATP agarose chromatography. See for example, Welch *et al.* (1985) Mol. Cell. Biol. 5:1229, the disclosure of which is incorporated herein by reference. Briefly, MgCl₂ is added to the previously isolated complex to a final concentration of 3mM.

- 29 -

Then, the complex is applied to an ATP agarose column (Sigma Chemical Co.) equilibrated in 20mM Tris-Acetate (pH 7.5), 20mM NaCl, 0.1mM EDTA, 15mM 2-mercaptoethanol, 3mM MgCl₂. The column is washed extensively with the equilibration buffer containing 0.5M NaCl, and then washed with buffer without the NaCl. Then the Hsp70 is eluted from the column with equilibration buffer containing 3mM ATP (Sigma Chemical Co.).

(c) Purification of Hsp90-peptide complexes.

10 A pellet of infected cells is resuspended in 3 volumes of 1X Lysis buffer consisting of 5mM sodium phosphate buffer (pH7), 150mM NaCl, 2mM CaCl₂, 2mM MgCl₂ and 1mM PMSF. The cell pellet is sonicated, on ice, until >95% cells are lysed as judged by microscopic examination. Alternatively, the cells may be lysed by mechanical shearing, as before.

15

The lysate is centrifuged at 1000g for 10 minutes to remove unbroken cells, nuclei and other debris. The supernatant from this centrifugation step subsequently is recentrifuged at 100,000g for 90 minutes.

20

Then, the supernatant is mixed for 2-3 hours at 4°C with Con A Sepharose equilibrated with PBS containing 2mM Ca²⁺ and 2mM Mg²⁺. When the cells are lysed by mechanical shearing, the supernatant is diluted with equal volume of 2X Lysis Buffer before proceeding. Then, the slurry is packed into a column and washed with 1X lysis buffer. The material that does not bind is dialyzed for 36 hours (three times, 100 volumes each time) against 20mM sodium phosphate pH 7.4, 1mM EDTA, 250mM NaCl, 1mM PMSF. The dialyzate is centrifuged at 17,000 rpm (Sorvall SS34 rotor) for 20 min. The resulting supernatant is applied to a Mono Q FPLC column (Pharmacia) equilibrated with lysis buffer and the bound proteins eluted with a salt gradient of 200mM to 600mM NaCl.

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- 30 -

The eluted fractions are analyzed by SDS-PAGE and the Hsp90 complexes identified by immunoblotting using an anti-Hsp90 antibody (for example, 3G3 from Affinity Bioreagents). Hsp90 can be purified to apparent homogeneity using this procedure. Approximately 150-200 µg of Hsp90 can be purified routinely from 1g of cells/tissue.

(d) Purification of gp96-peptide complexes.

10 A pellet of infected cells is resuspended in 4 volumes of buffer consisting of 30mM sodium bicarbonate buffer (pH7.5) and 1mM PMSF and the cells allowed to swell on ice for 20 min. The cell pellet then is homogenized in a Dounce homogenizer (the appropriate clearance of the homogenizer will vary according to each cells type) on ice until >95% cells are lysed.

15 The lysate is centrifuged at 1000g for 10 minutes to remove unbroken cells, nuclei and other debris. The supernatant from this centrifugation step then is recentrifuged at 100,000g for 90 minutes. The gp96-peptide complex can be purified either from the 100,000g pellet or from the supernatant.

20 When purified from the supernatant, the supernatant is diluted with equal volume of 2X Lysis Buffer and the supernatant mixed for 2-3 hours at 4°C with Con A Sepharose equilibrated with PBS containing 2mM Ca^{2+} and 2mM Mg^{2+} . Then, the slurry is packed into a column and washed with 1X lysis buffer until the OD_{280} drops to baseline. Then, the column is washed with 1/2 column bed volume of 10% α -methyl mannoside (α -MM) dissolved in PBS containing 2 mM Ca^{2+} and 2mM Mg^{2+} , the column sealed with a piece of parafilm, and incubated at 37°C for 15 min. Then the column is cooled to room temperature and the parafilm removed from the bottom of the column. Five column volumes of the α -MM buffer are

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- 31 -

applied to the column and the eluate analyzed by SDS-PAGE. Typically the resulting material is about 60 - 95% pure, however this depends upon the cell type and the tissue-to-lysis buffer ratio used. Then the sample is applied to a Mono Q FPLC column (Pharmacia) equilibrated with a buffer containing 5mM sodium phosphate, pH7. The proteins then are eluted from the column with a 0-1M NaCl gradient and the gp96 fraction elutes between 400mM and 550mM NaCl.

This procedure, however, may be modified by two additional steps, used either alone or in combination, to consistently produce apparently homogeneous gp96-peptide complexes. One optional step involves an ammonium sulfate precipitation prior to the Con A purification step and the other optional step involves DEAE-Sepharose purification after the Con A purification step but before the Mono Q FPLC step.

In the first optional step, the supernatant resulting from the 100,000g centrifugation step is brought to a final concentration of 50% ammonium sulfate by the addition ammonium sulfate. The ammonium sulfate is added slowly while gently stirring the solution in a beaker placed in a tray of ice water. The solution is stirred for about 2 to 12 h. at 4°C and the resulting solution centrifuged at 6,000 rpm (Sorvall SS34 rotor). The supernatant resulting from this step is removed, brought to 70% ammonium sulfate saturation by the addition of ammonium sulfate solution, and centrifuged at 6,000 rpm (Sorvall SS34 rotor). The resulting pellet from this step is harvested and suspended in PBS containing 70% ammonium sulfate in order to rinse the pellet. This mixture is centrifuged at 6,000 rpm (Sorvall SS34 rotor) and the pellet dissolved in PBS containing 2 mM Ca^{2+} and Mg^{2+} . Undissolved material is removed by a brief centrifugation at 15,000 rpm (Sorvall SS34 rotor). Then, the solution is mixed with Con A Sepharose and the procedure followed as before.

- 32 -

In the second optional step, the gp96 containing fractions eluted from the Con A column are pooled and the buffer exchanged for 5 mM sodium phosphate buffer, pH 7, 300 mM NaCl by dialysis, or preferably by buffer exchange on a Sephadex G25 column. After buffer exchange, the solution is mixed with DEAE-
5 Sepharose previously equilibrated with 5 mM sodium phosphate buffer, pH 7, 300 mM NaCl. The protein solution and the beads are mixed gently for 1 hour and poured into a column. Then, the column is washed with 5 mM sodium phosphate buffer, pH 7, 300 mM NaCl, until the absorbance at 280 nM drops to baseline. Then, the bound protein is eluted from the column with five volumes of 5 mM
10 sodium phosphate buffer, pH 7, 700 mM NaCl. Protein containing fractions are pooled and diluted with 5 mM sodium phosphate buffer, pH 7 in order to lower the salt concentration to 175 mM. The resulting material then is applied to the Mono Q FPLC column (Pharmacia) equilibrated with 5 mM sodium phosphate buffer, pH 7 and the protein that binds to the Mono Q FPLC column (Pharmacia) is
15 eluted as described before.

It is appreciated, however, that one skilled in the art may assess, by routine experimentation, the benefit of incorporating the optional steps into the purification protocol. In addition, it is appreciated also that the benefit of adding each of the
20 optional steps will depend upon the source of the starting material.

When the gp96 fraction is isolated from the 100,000g pellet, the pellet is suspended in 5 volumes of PBS containing either 1% sodium deoxycholate or 1% octyl glucopyranoside (but without the Mg^{2+} and Ca^{2+}) and incubated on ice for 1
25 h. The suspension is centrifuged at 20,000g for 30 min and the resulting supernatant dialyzed against several changes of PBS (also without the Mg^{2+} and Ca^{2+}) to remove the detergent. The dialysate is centrifuged at 100,000g for 90 min, the supernatant harvested, and calcium and magnesium are added to the supernatant to give final concentrations of 2mM, respectively. Then the sample is

- 33 -

purified by either the unmodified or the modified method for isolating gp96-peptide complex from the 100,000g supernatant, see above.

5 The gp96-peptide complexes can be purified to apparent homogeneity using this procedure. About 10-20 µg of gp96 can be isolated from 1g cells/tissue.

(e) Purification of HSP25 and HSP27.

10 The purification of Hsp25 and Hsp27 polypeptides has been disclosed previously and so is not discussed in detail herein. See for example Jakob et al. (1993) J. Biol. Chem. 268:1517-1520, the disclosure of which is incorporated herein by reference.

15 Briefly, the cell lysates are precipitated with 35% ammonium sulfate. The pellet is harvested by centrifugation, solubilized in buffer and fractionated by ion exchange chromatography using a DEAE Sepharose CL-6B column (Pharmacia Biotechnology, Inc.). The proteins are eluted with 50-200 mM NaCl gradient. The fractions containing Hsp25 and Hsp27 are identified by immunoblotting using suitable antibodies. The fractions are combined and fractionated by size exclusion
20 chromatography on a Superose 6 gel filtration column (Pharmacia).

(f) Purification of Hsp60.

25 The purification of Hsp60 has been discussed in detail previously and so is not discussed in detail herein. See for example, Vitanen et al. (1992) J. Biol. Chem. 267: 695-698, the disclosure of which is incorporated herein by reference.

Briefly, a mitochondrial matrix lysate is applied to a Mono Q FPLC column equilibrated with 50mM sodium phosphate, 1mM MgCl₂, 1mM EGTA, pH 6.9.

- 34 -

The proteins are eluted with a 0-1M NaCl gradient. The fractions containing Hsp65 are pooled and fractionated by ATP agarose chromatography as discussed above.

III. Preparation of Recombinant Stress Proteins

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It is contemplated that recombinant stress proteins and amino acid sequence variants thereof may be prepared using conventional recombinant DNA methodologies. For example, recombinant DNAs encoding either a known stress protein or a homologue can be inserted into a suitable host cell, the protein
10 expressed, harvested, renatured if necessary, and purified. Stress proteins currently known in the art are summarized in Table I, below.

The processes for manipulating, amplifying, and recombining DNA which encode amino acid sequences of interest are generally well known in the art, and
15 therefore, not described in detail herein. Methods of identifying and isolating genes encoding members of the stress protein families also are well understood, and are described in the patent and other literature.

Accordingly, the construction of DNAs encoding biosynthetic constructs as
20 disclosed herein can be performed using known techniques involving the use of various restriction enzymes which make sequence specific cuts in DNA to produce blunt ends or cohesive ends, DNA ligases, techniques enabling enzymatic addition of sticky ends to blunt-ended DNA, construction of synthetic DNAs by assembly of short or medium length oligonucleotides, cDNA synthesis techniques, and
25 synthetic probes for isolating genes of members of the stress protein families. Various promoter sequences and other regulatory DNA sequences used in achieving expression, and various types of host cells are also known and available. Conventional transfection techniques, and equally conventional techniques for cloning and subcloning DNA are useful in the practice of this invention and known

- 35 -

Table 1Families of Stress Proteins from Gething, et al., Infra

<u>Organism/Organelle</u>	<u>Hsp 60</u>	<u>Hsp 70</u>	<u>Hsp 90</u>
<u>E. coli</u>	GroEL	<u>DnaK</u>	HtpG (C62.5)
<u>Yeast</u>		<u>Ssa1-4p</u>	Hsp 83/Hsc83
/cytosol		<u>Kar2 (BiP)</u>	
/endoplasmic reticulum			
/mitochondria	Hsp 60 (Mif4p)	<u>Ssc1p</u>	
<u>Drosophila</u>		<u>Hsp 68</u>	
		<u>Hsp 70</u>	
		<u>Hsc 1.2.4</u>	
<u>Mammals</u>			
/cytosol		<u>Hsp 70 (p73)</u>	Hsp 90 (Hsp83)
		<u>Hsc 70 (p72)</u>	Hsp 87
/endoplasmic reticulum		<u>BiP (Grp 78)</u>	Grp 94 (Erp99)
			gp96
/mitochondria	Hsp 60 (Hsp 8)	<u>Hsp 70 (Grp 75)</u>	
<u>Plants</u>			
/endoplasmic reticulum		<u>b70 (BiP)</u>	
/chloroplasts	RUSBP		

Alternative names are shown in parentheses.

Detailed Description.

5 The invention is based on the discovery that a stress protein-peptide complex when isolated from a eukaryotic cell infected with a preselected intracellular pathogen and then administered to a mammal can stimulate a cytotoxic T cell response directed against cells infected with the same pathogen. This discovery provides a significant advance to the field of vaccine development.

10 In accordance with the invention, the aforementioned discovery is exploited to provide a family of vaccines which may be used to immunize mammals against diseases caused by intracellular pathogens. In principle, the vaccines can be prepared against any intracellular pathogen of interest, for example: viruses; bacteria; protozoa; fungi; or intracellular parasites. Generic methodologies useful
15 for preparing vaccines against all of these classes of pathogens are discussed in detail hereinbelow.

As will be appreciated by those skilled in the art, the stress protein-peptide vaccines described herein have several advantages over the vaccines currently
20 available. First, the stress protein-peptide vaccines provide an alternative approach for stimulating cellular immunity and obviate the use of intact intracellular (attenuated or otherwise) pathogens. Second, since the vaccines do not contain intact organisms this reduces the risk of causing the disease the vaccine was designed to induce immunity against. Third, the vaccines described herein are
25 ideal for inducing immune responses against either defined antigenic determinants isolated from an intracellular pathogen or as yet undefined antigenic determinants. Furthermore, vaccines may be prepared that are effective against pathogens that normally evade the immune system by evolving new antigenic coat proteins, i.e., the influenza virus. Fourth, vaccines of this type may in principle be prepared

- 20 -

against any intracellular pathogen of interest. Fifth, the vaccines may be prepared synthetically using the methodologies described hereinafter thereby providing completely defined vaccines that are suitable for administration to humans.

5 It is contemplated that the vaccines may be administered either prophylactically or therapeutically. When administered prophylactically the vaccine may stimulate in the mammal a cytotoxic T cell response that permits the vaccinee to resist subsequent infection by the intracellular pathogen. Alternatively, when administered therapeutically the vaccine may stimulate in the mammal a
10 cytotoxic T cell response against a pathogen which is presently infecting and causing disease in the mammal.

 The specific component of the vaccine that induces in the recipient a specific cytotoxic T cell response against the pathogen is a stress protein-peptide complex.
15 The peptide may be any amino acid sequence that is present in a eukaryotic cell infected with an intracellular pathogen but which is not present when such a cell is not infected with the same pathogen. This includes peptides that not only originate from the pathogen itself but also are synthesized by the infected cell in response to infection by the intracellular pathogen.

20 The immunogenic complexes may be purified from any eukaryotic cell, including: whole tissues; isolated cells; and immortalized eukaryotic cell lines infected with the intracellular pathogen. The complexes may be purified by using conventional protein purification techniques well known in the art. For example, it
25 is contemplated that an immunogenic complex capable of stimulating a cytotoxic T cell response against the influenza virus may be harvested from a eukaryotic cell line that is infected with the influenza virus.

- 21 -

In addition, it has been found that the peptide can be eluted from the stress protein-complex either in the presence of ATP or low pH. Neither the peptide nor the stress protein individually are effective at inducing a cytotoxic T cell response. These experimental conditions, however, may be exploited to isolate peptides from infected cells which may contain potentially useful antigenic determinants. Once isolated, the amino acid sequence of each antigenic peptide may be determined using conventional amino acid sequencing methodologies. Consequently, the antigenic determinants for potentially any intracellular pathogen of interest can be identified readily using the methodologies described herein. As discussed in detail hereinafter, this property may be exploited in the preparation of completely synthetic vaccines.

Similarly, it has been found that potentially immunogenic peptides may be eluted from MHC-peptide complexes using techniques well known in the art. See for example, Falk *et al.* (1990) Nature 343:248-251; Rotzsche *et al.* (1990) Nature 348:252-254; Elliott *et al.* (1990) Nature 348:195-197; Falk *et al.* (1991) Nature 351:290-296; Demotz *et al.* (1989) Nature 334:682-684; Rotzsche *et al.* (1990) Science 249:283-287, the disclosures of which are incorporated herein by reference. Although the peptides eluted from the MHC complexes may define a potentially protective antigenic determinant, it is appreciated that administration of the isolated peptide in a conventional subunit vaccine may be ineffective at stimulating a cytotoxic T cell response in the recipient. Consequently, it is contemplated that the peptides eluted from MHC-peptide complexes may be reconstituted with a stress protein, using the methodologies described herein, thereby to generate a stress protein-peptide complex effective at stimulating a cytotoxic T cell response capable of targeting and lysing cells expressing the antigenic peptide.

Stress proteins useful in the practice of the instant invention may be defined as any cellular protein that satisfies the following criteria. It is a protein whose

- 22 -

intracellular concentration increases when a cell is exposed to a stressful stimuli, it is capable of binding other proteins or peptides, and it is capable of releasing the bound proteins or peptides in the presence of adenosine triphosphate (ATP) or low pH.

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The first stress proteins to be identified were the heat shock proteins (Hsp). As their name implies, Hsps are synthesized by a cell in response to heat shock. To date, three major families of Hsp have been identified based on molecular weight. The families have been called Hsp60, Hsp70 and Hsp90 where the numbers reflect the approximate molecular weight of the stress proteins in kD. Many members of these families subsequently were found to be induced in response to other stressful stimuli including, but not limited to, nutrient deprivation, metabolic disruption, oxygen radicals, and infection with intracellular pathogens. See for example: Welch (May 1993) Scientific American 56-64; Young (1990) Annu. Rev. Immunol. 8:401-420; Craig (1993) Science 260:1902-1903; Gething et al. (1992) Nature 355:33-45; and Lindquist et al. (1988) Annu. Rev. Genetics 22:631-677, the disclosures of which are incorporated herein by reference. Accordingly, it is contemplated that stress proteins belonging to all three families may be useful in the practice of the instant invention.

20

The major stress proteins can accumulate to very high levels in stressed cells, but they occur at low to moderate levels in cells that have not been stressed. For example, the highly inducible mammalian Hsp70 is hardly detectable at normal temperatures but becomes one of the most actively synthesized proteins in the cell upon heat shock (Welch et al. (1985), J. Cell. Biol. 101:1198-1211). In contrast, Hsp90 and Hsp60 proteins are abundant at normal temperatures in most, but not all, mammalian cells and are further induced by heat (Lai et al. (1984), Mol. Cell. Biol. 4:2802-10; van Bergen en Henegouwen et al. (1987), Genes Dev. 1:525-531).

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- 23 -

Stress proteins are among the most highly conserved proteins in existence. For example, DnaK, the Hsp70 from E. coli has about 50% amino acid sequence identity with Hsp70 proteins from eukaryotes (Bardwell et al. (1984) Proc. Natl. Acad. Sci. 81:848-852). The Hsp60 and Hsp90 families also show similarly high levels of intrafamilial conservation (Hickey et al. (1989) Mol. Cell Biol. 9:2615-2626; Jindal (1989) Mol. Cell. Biol. 9:2279-2283). In addition, it has been discovered that the Hsp60, Hsp70 and Hsp90 families are composed of proteins that are related to the stress proteins in sequence, for example, having greater than 35% amino acid identity, but whose expression levels typically remain unaltered under conditions stressful to the host cell. An example of such a protein includes the constitutively expressed cystolic protein Hsc 70 which is related in amino acid sequence to the stress-induced protein Hsp 70. It is, therefore, contemplated that the definition of stress protein, as used herein, embraces other proteins, muteins, analogs, and variants thereof having at least 35% to 55%, preferably 55% to 75%, and most preferably 75% to 95% amino acid identity with members of the three families whose expression levels in a cell are enhanced in response to a stressful stimulus. The purification of stress proteins belonging to these three families is described below.

The immunogenic stress protein-peptide complexes of the invention may include any complex containing a stress protein and a peptide that is capable of inducing an immune response in a mammal. The peptides preferably are non covalently associated with the stress protein. Preferred complexes may include, but are not limited to, Hsp60-peptide, Hsp70-peptide and Hsp90-peptide complexes. For example, a stress protein called gp96 which is present in the endoplasmic reticulum of eukaryotic cells and is related to the cytoplasmic Hsp90s can be used to generate an effective vaccine containing a gp96-peptide complex.

- 24 -

Another family of low molecular weight heat shock proteins has now been identified and is called Hsp 25/Hsp 27. The purification of these proteins is discussed below. It is contemplated that these low molecular weight proteins may also have utility in the instant invention.

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It has been discovered also that the stress protein-peptide complexes of the invention can be prepared from cells infected with an intracellular pathogen as well as cells that have been transformed by an intracellular pathogen. For example, immunogenic stress protein peptide complexes may be isolated from eukaryotic cells transformed with a transforming virus such as SV40, see below.

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In a preferred aspect of the invention, the purified stress protein-peptide vaccines may have particular utility in the treatment of human diseases caused by intracellular pathogens. It is appreciated, however, that the vaccines developed using the principles described herein will be useful in treating diseases of other mammals, for example, farm animals including: cattle; horses; sheep; goats; and pigs, and household pets including: cats; and dogs, that similarly are caused by intracellular pathogens.

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In accordance with the methods described herein, vaccines may be prepared that stimulate cytotoxic T cell responses against cells infected with viruses including, but not limited to, hepatitis type A, hepatitis type B, hepatitis type C, influenza, varicella, adenovirus, HSV-I, HSV-II, rinderpest rhinovirus, echovirus, rotavirus, respiratory syncytial virus, papilloma virus, papova virus, cytomegalovirus, echinovirus, arbovirus, huntavirus, coxsachie virus, mumps virus, measles virus, rubella virus, polio virus, HIV-I, and HIV-II. Similarly, vaccines may also be prepared that stimulate cytotoxic T cell responses against cells infected with intracellular bacteria, including, but not limited to, *Mycobacteria*, *Rickettsia*, *Mycoplasma*, *Neisseria* and *Legionella*. In addition, vaccines may also be

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- 25 -

prepared that stimulate cytotoxic T cell responses against cells infected with intracellular protozoa, including, but not limited to, *Leishmania*, *Kokzidioa*, and *Trypanosoma*. Furthermore, vaccines may be prepared that stimulate cytotoxic T cell responses against cells infected with intracellular parasites including, but not
5 limited to, *Chlamydia* and *Rickettsia*.

I. Propagation of infected eukaryotic cells.

As will be appreciated by those skilled in the art, the protocols described
10 herein may be used to isolate stress protein-peptide complexes from any eukaryotic cell, for example, tissues, isolated cells or immortalized eukaryotic cell lines infected with a preselected intracellular pathogen.

When immortalized animal cell lines are used as a source of the stress
15 protein-peptide complex it is of course important to use cell lines that can be infected with the pathogen of interest. In addition, it is preferable to use cells that are derived from the same species as the intended recipient of the vaccine.

For example, in order to prepare a stress protein-peptide complex for
20 administration to humans that may be effective against HIV-I, the virus may be propagated in human cells which include, but are not limited to, human CD4+ T cells, HepG2 cells, and U937 promonocytic cells. In order to prepare a stress protein-peptide complex for administration to humans that may be effective against HIV-II, the virus may be propagated in, for example, human CD4+ T cells.
25 Similarly, influenza viruses may be propagated in, for example, human fibroblast cell lines and MDCK cells, and mycobacteria may be cultured in, for example, human Schwaan cells.

- 26 -

If the intracellular pathogens do not lyse the infected cells then the infected cells are cultured under the same conditions as the normal uninfected cells. For example, mycobacteria may be propagated in nerve cultures of the sensory ganglia of newborn Swiss white mice. The nerve cells are cultured in a growth medium
5 containing 70% Dulbecco modified Eagle minimal essential medium (DMEM) with 0.006% glucose, 20% fetal calf serum, 10% chicken embryo extract and cytosine arabinoside. After eight to ten days, the cultures are inoculated with $5-8 \times 10^6$ mycobacteria isolated from fresh nodules of untreated lepromatous leprosy patients. The infected cells may be cultured at 37°C , for up to 6 weeks, after which
10 the infected cells are harvested and the stress protein-peptide complexes isolated. See for example, Mukherjee *et al.* (1985) J. Clin. Micro. 21:808-814, the disclosure of which is incorporated herein by reference.

If, on the other hand, the host cells are lysed by the pathogen of interest (as
15 in the case of influenza virus) the cells may still be grown under standard conditions except the cells are washed and harvested just prior to lysis of the host cell. For example, during the purification of stress protein-peptide complexes from influenza infected cells, fibroblasts (or other cell types) are infected for 1 hour at 37°C with 5-10 plaque forming units (PFU) of virus per cell. The infected cells may
20 be cultured in plain DMEM medium for 24 hours at 37°C . After 24 hours the cells are washed and harvested prior to lysis. The stress protein-peptide complexes may be isolated using the procedures set forth below.

In addition, when the gene encoding a particular antigenic determinant has
25 been identified, the gene of interest may be transfected and expressed in an immortalized human or other mammalian cell line using techniques well known in the art. See for example "Current Protocols in Molecular Biology" (1989), eds. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA and Struhl K, Wiley Interscience, the disclosure of which is incorporated by reference herein. The

- 27 -

transfected cells may be grown under standard conditions and the complexes isolated subsequently.

II. Preparation of Stress Proteins and Immunogenic Stress Protein-peptide complexes.

Methods for preparing Hsp70-peptide complexes, Hsp90-peptide complexes, gp96-peptide complexes, Hsp70, Hsp25/Hsp27, and Hsp60 are set forth below.

(a) Purification of Hsp70-peptide complexes.

A pellet of infected cells is resuspended in 3 volumes of 1X Lysis buffer consisting of 5mM sodium phosphate buffer (pH7), 150mM NaCl, 2mM CaCl₂, 2mM MgCl₂ and 1mM phenyl methyl sulfonyl fluoride (PMSF). The pellet is sonicated, on ice, until >99% cells are lysed as judged by microscopic examination. Alternatively, the cells may be lysed by mechanical shearing. In this procedure, the cells are resuspended in 30mM sodium bicarbonate pH 7.5, 1mM PMSF, incubated on ice for 20 min. and then homogenized in a dounce homogenizer until >95% cells are lysed.

The lysate is centrifuged at 1000g for 10 minutes to remove unbroken cells, nuclei and other debris. The supernatant from this centrifugation step is then recentrifuged at 100,000g for 90 minutes.

The supernatant is mixed for 2-3 hours at 4°C with Con A Sepharose equilibrated with PBS containing 2mM Ca²⁺ and 2mM Mg²⁺. When the cells are lysed by mechanical shearing, the supernatant is diluted with equal volume of 2X Lysis Buffer before proceeding. Then the slurry is packed into a column and

- 28 -

washed with 1X lysis buffer. The material that does not bind is dialyzed for 36 hours (three times, 100 volumes each time) against 10mM Tris-Acetate pH 7.5, 0.1 mM EDTA, 10mM NaCl, 1mM PMSF. The dialyzate is centrifuged for 20 min. at 17,000 rpm (Sorvall SS34 rotor) and the resulting supernatant applied to a Mono Q FPLC column (Pharmacia) equilibrated in 20mM Tris-Acetate pH 7.5, 20 mM NaCl, 0.1mM EDTA and 15mM 2-mercaptoethanol. Then the proteins are eluted with a 20 mM to 500 mM NaCl gradient. The fractions are characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting using an appropriate anti-Hsp70 antibody (such as clone N27F3-4 from StressGen).

The fractions that are strongly immunoreactive with the antibody are pooled and the Hsp70-peptide complexes precipitated with ammonium sulfate. The complex is precipitated in the 50%-70% ammonium sulfate cut. The protein pellet is harvested by centrifugation at 17,000 rpm (SS34 Sorvall rotor) and washed with 70% ammonium sulfate. Then the pellet is solubilized and the residual ammonium sulfate removed by gel filtration on a Sephadex® G25 column (Pharmacia).

The Hsp70-peptide complex can be purified to apparent homogeneity using this method. Up to 1mg of Hsp70-peptide complex can be purified from 1g of cells/tissue.

(b) Purification of Hsp70.

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The Hsp70 polypeptide may be purified from the Hsp70-peptide complex by ATP agarose chromatography. See for example, Welch *et al.* (1985) *Mol. Cell. Biol.* 5:1229, the disclosure of which is incorporated herein by reference. Briefly, $MgCl_2$ is added to the previously isolated complex to a final concentration of 3mM.

- 29 -

Then, the complex is applied to an ATP agarose column (Sigma Chemical Co.) equilibrated in 20mM Tris-Acetate (pH 7.5), 20mM NaCl, 0.1mM EDTA, 15mM 2-mercaptoethanol, 3mM MgCl₂. The column is washed extensively with the equilibration buffer containing 0.5M NaCl, and then washed with buffer without the NaCl. Then the Hsp70 is eluted from the column with equilibration buffer containing 3mM ATP (Sigma Chemical Co.).

(c) Purification of Hsp90-peptide complexes.

10 A pellet of infected cells is resuspended in 3 volumes of 1X Lysis buffer consisting of 5mM sodium phosphate buffer (pH7), 150mM NaCl, 2mM CaCl₂, 2mM MgCl₂ and 1mM PMSF. The cell pellet is sonicated, on ice, until >95% cells are lysed as judged by microscopic examination. Alternatively, the cells may be lysed by mechanical shearing, as before.

15 The lysate is centrifuged at 1000g for 10 minutes to remove unbroken cells, nuclei and other debris. The supernatant from this centrifugation step subsequently is recentrifuged at 100,000g for 90 minutes.

20 Then, the supernatant is mixed for 2-3 hours at 4°C with Con A Sepharose equilibrated with PBS containing 2mM Ca²⁺ and 2mM Mg²⁺. When the cells are lysed by mechanical shearing, the supernatant is diluted with equal volume of 2X Lysis Buffer before proceeding. Then, the slurry is packed into a column and washed with 1X lysis buffer. The material that does not bind is dialyzed for 36
25 hours (three times, 100 volumes each time) against 20mM sodium phosphate pH 7.4, 1mM EDTA, 250mM NaCl, 1mM PMSF. The dialyzate is centrifuged at 17,000 rpm (Sorvall SS34 rotor) for 20 min. The resulting supernatant is applied to a Mono Q FPLC column (Pharmacia) equilibrated with lysis buffer and the bound proteins eluted with a salt gradient of 200mM to 600mM NaCl.

- 30 -

The eluted fractions are analyzed by SDS-PAGE and the Hsp90 complexes identified by immunoblotting using an anti-Hsp90 antibody (for example, 3G3 from Affinity Bioreagents). Hsp90 can be purified to apparent homogeneity using this procedure. Approximately 150-200 μ g of Hsp90 can be purified routinely from 1g of cells/tissue.

(d) Purification of gp96-peptide complexes.

10 A pellet of infected cells is resuspended in 4 volumes of buffer consisting of 30mM sodium bicarbonate buffer (pH7.5) and 1mM PMSF and the cells allowed to swell on ice for 20 min. The cell pellet then is homogenized in a Dounce homogenizer (the appropriate clearance of the homogenizer will vary according to each cells type) on ice until >95% cells are lysed.

15

The lysate is centrifuged at 1000g for 10 minutes to remove unbroken cells, nuclei and other debris. The supernatant from this centrifugation step then is recentrifuged at 100,000g for 90 minutes. The gp96-peptide complex can be purified either from the 100,000g pellet or from the supernatant.

20

When purified from the supernatant, the supernatant is diluted with equal volume of 2X Lysis Buffer and the supernatant mixed for 2-3 hours at 4°C with Con A Sepharose equilibrated with PBS containing 2mM Ca^{2+} and 2mM Mg^{2+} . Then, the slurry is packed into a column and washed with 1X lysis buffer until the OD₂₈₀ drops to baseline. Then, the column is washed with 1/2 column bed volume of 10% α -methyl mannoside (α -MM) dissolved in PBS containing 2 mM Ca^{2+} and 2mM Mg^{2+} , the column sealed with a piece of parafilm, and incubated at 37°C for 15 min. Then the column is cooled to room temperature and the parafilm removed from the bottom of the column. Five column volumes of the α -MM buffer are

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- 31 -

applied to the column and the eluate analyzed by SDS-PAGE. Typically the resulting material is about 60 - 95% pure, however this depends upon the cell type and the tissue-to-lysis buffer ratio used. Then the sample is applied to a Mono Q FPLC column (Pharmacia) equilibrated with a buffer containing 5mM sodium phosphate, pH7. The proteins then are eluted from the column with a 0-1M NaCl gradient and the gp96 fraction elutes between 400mM and 550mM NaCl.

This procedure, however, may be modified by two additional steps, used either alone or in combination, to consistently produce apparently homogeneous gp96-peptide complexes. One optional step involves an ammonium sulfate precipitation prior to the Con A purification step and the other optional step involves DEAE-Sepharose purification after the Con A purification step but before the Mono Q FPLC step.

In the first optional step, the supernatant resulting from the 100,000g centrifugation step is brought to a final concentration of 50% ammonium sulfate by the addition ammonium sulfate. The ammonium sulfate is added slowly while gently stirring the solution in a beaker placed in a tray of ice water. The solution is stirred for about 2 to 12 h. at 4°C and the resulting solution centrifuged at 6,000 rpm (Sorvall SS34 rotor). The supernatant resulting from this step is removed, brought to 70% ammonium sulfate saturation by the addition of ammonium sulfate solution, and centrifuged at 6,000 rpm (Sorvall SS34 rotor). The resulting pellet from this step is harvested and suspended in PBS containing 70% ammonium sulfate in order to rinse the pellet. This mixture is centrifuged at 6,000 rpm (Sorvall SS34 rotor) and the pellet dissolved in PBS containing 2 mM Ca^{2+} and Mg^{2+} . Undissolved material is removed by a brief centrifugation at 15,000 rpm (Sorvall SS34 rotor). Then, the solution is mixed with Con A Sepharose and the procedure followed as before.

- 32 -

In the second optional step, the gp96 containing fractions eluted from the Con A column are pooled and the buffer exchanged for 5 mM sodium phosphate buffer, pH 7, 300 mM NaCl by dialysis, or preferably by buffer exchange on a Sephadex G25 column. After buffer exchange, the solution is mixed with DEAE-
5 Sepharose previously equilibrated with 5 mM sodium phosphate buffer, pH 7, 300 mM NaCl. The protein solution and the beads are mixed gently for 1 hour and poured into a column. Then, the column is washed with 5 mM sodium phosphate buffer, pH 7, 300 mM NaCl, until the absorbance at 280 nm drops to baseline. Then, the bound protein is eluted from the column with five volumes of 5 mM
10 sodium phosphate buffer, pH 7, 700 mM NaCl. Protein containing fractions are pooled and diluted with 5 mM sodium phosphate buffer, pH 7 in order to lower the salt concentration to 175 mM. The resulting material then is applied to the Mono Q FPLC column (Pharmacia) equilibrated with 5 mM sodium phosphate buffer, pH 7 and the protein that binds to the Mono Q FPLC column (Pharmacia) is
15 eluted as described before.

It is appreciated, however, that one skilled in the art may assess, by routine experimentation, the benefit of incorporating the optional steps into the purification protocol. In addition, it is appreciated also that the benefit of adding each of the
20 optional steps will depend upon the source of the starting material.

When the gp96 fraction is isolated from the 100,000g pellet, the pellet is suspended in 5 volumes of PBS containing either 1% sodium deoxycholate or 1% octyl glucopyranoside (but without the Mg^{2+} and Ca^{2+}) and incubated on ice for 1
25 h. The suspension is centrifuged at 20,000g for 30 min and the resulting supernatant dialyzed against several changes of PBS (also without the Mg^{2+} and Ca^{2+}) to remove the detergent. The dialysate is centrifuged at 100,000g for 90 min, the supernatant harvested, and calcium and magnesium are added to the supernatant to give final concentrations of 2mM, respectively. Then the sample is

- 33 -

purified by either the unmodified or the modified method for isolating gp96-peptide complex from the 100,000g supernatant, see above.

The gp96-peptide complexes can be purified to apparent homogeneity using
5 this procedure. About 10-20 µg of gp96 can be isolated from 1g cells/tissue.

(e) Purification of HSP25 and HSP27.

The purification of Hsp25 and Hsp27 polypeptides has been disclosed
10 previously and so is not discussed in detail herein. See for example Jakob et al.
(1993) J. Biol. Chem. 268:1517-1520, the disclosure of which is incorporated herein
by reference.

Briefly, the cell lysates are precipitated with 35% ammonium sulfate. The
15 pellet is harvested by centrifugation, solubilized in buffer and fractionated by ion
exchange chromatography using a DEAE Sepharose CL-6B column (Pharmacia
Biotechnology, Inc.). The proteins are eluted with 50-200 mM NaCl gradient. The
fractions containing Hsp25 and Hsp27 are identified by immunoblotting using
suitable antibodies. The fractions are combined and fractionated by size exclusion
20 chromatography on a Superose 6 gel filtration column (Pharmacia).

(f) Purification of Hsp60.

The purification of Hsp60 has been discussed in detail previously and so is
25 not discussed in detail herein. See for example, Vitanen et al. (1992) J. Biol. Chem.
267: 695-698, the disclosure of which is incorporated herein by reference.

Briefly, a mitochondrial matrix lysate is applied to a Mono Q FPLC column
equilibrated with 50mM sodium phosphate, 1mM MgCl₂, 1mM EGTA, pH 6.9.

- 34 -

The proteins are eluted with a 0-1M NaCl gradient. The fractions containing Hsp65 are pooled and fractionated by ATP agarose chromatography as discussed above.

III. Preparation of Recombinant Stress Proteins

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It is contemplated that recombinant stress proteins and amino acid sequence variants thereof may be prepared using conventional recombinant DNA methodologies. For example, recombinant DNAs encoding either a known stress protein or a homologue can be inserted into a suitable host cell, the protein
10 expressed, harvested, renatured if necessary, and purified. Stress proteins currently known in the art are summarized in Table I, below.

The processes for manipulating, amplifying, and recombining DNA which encode amino acid sequences of interest are generally well known in the art, and
15 therefore, not described in detail herein. Methods of identifying and isolating genes encoding members of the stress protein families also are well understood, and are described in the patent and other literature.

Accordingly, the construction of DNAs encoding biosynthetic constructs as
20 disclosed herein can be performed using known techniques involving the use of various restriction enzymes which make sequence specific cuts in DNA to produce blunt ends or cohesive ends, DNA ligases, techniques enabling enzymatic addition of sticky ends to blunt-ended DNA, construction of synthetic DNAs by assembly of short or medium length oligonucleotides, cDNA synthesis techniques, and
25 synthetic probes for isolating genes of members of the stress protein families. Various promoter sequences and other regulatory DNA sequences used in achieving expression, and various types of host cells are also known and available. Conventional transfection techniques, and equally conventional techniques for cloning and subcloning DNA are useful in the practice of this invention and known

- 35 -

Table 1Families of Stress Proteins from Gething, et al., Infra

<u>Organism/Organelle</u>	<u>Hsp 60</u>	<u>Hsp 70</u>	<u>Hsp 90</u>
<u>E. coli</u>	GroEL	<u>DnaK</u>	HtpG (C62.5)
<u>Yeast</u>		<u>Ssa1-4p</u>	Hsp 83/Hsc83
/cytosol		<u>Karp2 (BiP)</u>	
/endoplasmic reticulum			
/mitochondria	Hsp 60 (Mif4p)	<u>Ssc1p</u>	
<u>Drosophila</u>		<u>Hsp 68</u>	
		<u>Hsp 70</u>	
		<u>Hsc 1.2.4</u>	
<u>Mammals</u>		<u>Hsp 70 (p73)</u>	Hsp 90 (Hsp83)
/cytosol		<u>Hsc 70 (p72)</u>	Hsp 87
/endoplasmic reticulum		<u>BiP (Grp 78)</u>	Grp 94 (Erp99) gp96
/mitochondria	Hsp 60 (Hsp E)	<u>Hsp 70 (Grp 75)</u>	
<u>Plants</u>			
/endoplasmic reticulum		<u>b70 (BiP)</u>	
/chloroplasts	RUSBP		

Alternative names are shown in parentheses.

- 36 -

to those skilled in the art. Various types of vectors may be used such as plasmids and viruses including animal viruses and bacteriophages. The vectors may exploit various marker genes which impart to a successfully transfected cell a detectable phenotypic property that can be used to identify which of a family of clones has successfully incorporated the recombinant DNA of the vector.

DNA molecules encoding potentially useful stress proteins may be obtained by a variety of methods. Genes of interest may be purified from standard cDNA libraries using colony or plaque hybridization technologies or by using polymerase chain reaction (PCR) methodologies, all of which are well known in the art. See for example, "Molecular Cloning: A Laboratory Manual, 2nd Edition" Sambrook et al. (1989), Cold Spring Harbor Press, the disclosure of which is incorporated herein by reference. Alternatively, the preferred genes can be generated by the assembly of synthetic oligonucleotides produced in a conventional, automated, polynucleotide synthesizer followed by ligation with appropriate ligases. For example, overlapping, complementary DNA fragments comprising 15 bases may be synthesized semi manually using phosphoramidite chemistry, with end segments left unphosphorylated to prevent polymerization during ligation. One end of the synthetic DNA is left with a "sticky end" corresponding to the site of action of a particular restriction endonuclease, and the other end is left with an end corresponding to the site of action of another restriction endonuclease. Alternatively, this approach can be fully automated. The DNA encoding the biosynthetic constructs may be created by synthesizing longer single strand fragments (e.g., 50-100 nucleotides long) in, for example, an Applied Biosystems oligonucleotide synthesizer, and then ligating the fragments.

The recombinant DNA constructs then may be integrated into an expression vector and transfected into an appropriate host cell for protein expression. Useful host cells include E. coli, Saccharomyces, the insect/baculovirus cell system,

- 37 -

myeloma cells, and various other mammalian cells. In E. coli and other microbial hosts, the synthetic genes can be expressed as fusion proteins. Expression in eukaryotes can be accomplished by the transfection of DNA sequences encoding the biosynthetic protein of interest into myeloma or other type of cell line.

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The vector additionally may include various sequences to promote correct expression of the recombinant protein, including transcriptional promoter and termination sequences, enhancer sequences, preferred ribosome binding site sequences, preferred mRNA leader sequences, preferred protein processing sequences, preferred signal sequences for protein secretion, and the like. The DNA sequence encoding the gene of interest also may be manipulated to remove potentially inhibiting sequences or to minimize unwanted secondary structure formation. The recombinant protein also may be expressed as a fusion protein. After being translated, the protein may be purified from the cells themselves or recovered from the culture medium.

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For example, if the gene is to be expressed in E. coli, it may first be cloned into an expression vector. This is accomplished by positioning the engineered gene downstream of a promoter sequence such as Trp or Tac, and a gene coding for a leader peptide such as fragment B of protein A (FB). The resulting fusion proteins accumulate in refractile bodies in the cytoplasm of the cells, and may be harvested after disruption of the cells by French press or sonication. The refractile bodies are solubilized, and the expressed proteins refolded and cleaved by methods already established for many other recombinant proteins.

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Expression of the engineered genes in eukaryotic cells requires the establishment of appropriate cells and cell lines that are easy to transfect, are capable of stably maintaining foreign DNA with an unarranged sequence, and which have the necessary cellular components for efficient transcription,

- 38 -

translation, post-translation modification, and secretion of the protein. In addition, a suitable vector carrying the gene of interest also is necessary. DNA vector design for transfection into mammalian cells should include appropriate sequences to promote expression of the gene of interest as described supra, including

5 appropriate transcription initiation, termination, and enhancer sequences, as well as sequences that enhance translation efficiency, such as the Kozak consensus sequence. Preferred DNA vectors also include a marker gene and means for amplifying the copy number of the gene of interest. A detailed review of the state of the art of the production of foreign proteins in mammalian cells, including useful

10 cells, protein expression-promoting sequences, marker genes, and gene amplification methods, is disclosed in Genetic Engineering 7:91-127 (1988).

The best-characterized transcription promoters useful for expressing a foreign gene in a particular mammalian cell are the SV40 early promoter, the

15 adenovirus promoter (AdMLP), the mouse metallothionein-I promoter (mMT-I), the Rous sarcoma virus (RSV) long terminal repeat (LTR), the mouse mammary tumor virus long terminal repeat (MMTV-LTR), and the human cytomegalovirus major intermediate-early promoter (hCMV). The DNA sequences for all of these promoters are known in the art and are available commercially.

20 The use of a selectable DHFR gene in a dhfr^r cell line is a well characterized method useful in the amplification of genes in mammalian cell systems. Briefly, the DHFR gene is provided on the vector carrying the gene of interest, and addition of increasing concentrations of the cytotoxic drug methotrexate leads to amplification

25 of the DHFR gene copy number, as well as that of the associated gene of interest. DHFR as a selectable, amplifiable marker gene in transfected Chinese hamster ovary cell lines (CHO cells) is particularly well characterized in the art. Other useful amplifiable marker genes include the adenosine deaminase (ADA) and glutamine synthetase (GS) genes.

- 39 -

The choice of cells/cell lines is also important and depends on the needs of the experimenter. Monkey kidney cells (COS) provide high levels of transient gene expression, providing a useful means for rapidly testing vector construction and the expression of cloned genes. COS cells are transfected with a simian virus 40 (SV40) vector carrying the gene of interest. The transfected COS cells eventually die, thus preventing the long term production of the desired protein product. However, transient expression does not require the time consuming process required for the development of a stable cell line. Among established cell lines, CHO cells may be the best-characterized to date. CHO cells are capable of expressing proteins from a broad range of cell types. The general applicability of CHO cells and its successful production for a wide variety of human proteins in unrelated cell types emphasizes the underlying similarity of all mammalian cells.

The various cells, cell lines and DNA sequences that can be used for mammalian cell expression of the recombinant stress protein constructs of the invention are well characterized in the art and are readily available. Other promoters, selectable markers, gene amplification methods and cells also may be used to express the proteins of this invention. Particular details of the transfection, expression, and purification of recombinant proteins are well documented in the art and are understood by those having ordinary skill in the art. Further details on the various technical aspects of each of the steps used in recombinant production of foreign genes in mammalian cell expression systems can be found in a number of texts and laboratory manuals in the art, such as, for example, Current Protocols in Molecular Biology, (1989) eds. Ausubel *et al.*, Wiley Interscience.

- 40 -

IV. Isolation of Potentially Immunogenic Peptides.

As mentioned previously, potentially immunogenic peptides may be isolated from either stress protein-peptide complexes or MHC-peptide complexes.

5 Protocols for isolating peptides from either of these complexes are set forth below.

(a) Peptides from Stress Protein-peptide Complexes.

Two methods may be used to elute the peptide from a stress protein-peptide complex. One approach involves incubating the stress protein-peptide complex in the presence of ATP, the other involves incubating the complexes in a low pH buffer.

Briefly, the complex of interest is centrifuged through a Centricon 10 assembly (Millipore) to remove any low molecular weight material loosely associated with the complex. The large molecular weight fraction may be removed and analyzed by SDS-PAGE while the low molecular weight may be analyzed by HPLC as described below. In the ATP incubation protocol, the stress protein-peptide complex in the large molecular weight fraction is incubated with 10mM ATP for 30 minutes at room temperature. In the low pH protocol, acetic acid is added to the stress protein-peptide complex to give a final concentration of 10% (vol/vol) and the mixture incubated in a boiling water bath for 10 minutes. See for example, Van Bleek *et al.* (1990) *Nature* 348:213-216; and Li *et al.* (1993) *EMBO Journal* 12:3143-3151, the disclosures of which are incorporated herein by reference.

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The resulting samples are centrifuged through an Centricon 10 assembly as mentioned previously. The high and low molecular weight fractions are recovered. The remaining large molecular weight stress protein-peptide complexes can be reincubated with ATP or low pH to remove any remaining peptides.

- 41 -

The resulting lower molecular weight fractions are pooled, concentrated by evaporation and dissolved in 0.1% trifluoroacetic acid (TFA). Then, the dissolved material is fractionated by reverse phase high pressure liquid chromatography (HPLC), using for example a VYDAC C18 reverse phase column equilibrated with 0.1% TFA. The bound material subsequently is eluted by developing the column with a linear gradient of 0 to 80% acetonitrile in 0.1% TFA at a flow rate of about 0.8 ml/min. The elution of the peptides can be monitored by OD₂₁₀ and the fractions containing the peptides collected.

(b) Peptides from MHC-peptide Complexes.

The isolation of potentially immunogenic peptides from MHC molecules is well known in the art and so is not described in detail herein. See for example, Falk *et al.* (1990) *Nature* 348:248-251; Rotzsche *et al.* (1990) *Nature* 348:252-254; Elliott *et al.* (1990) *Nature* 348:195-197; Falk *et al.* (1991) *Nature* 351:290-296, Demotz *et al.* (1989) *Nature* 343:682-684; Rotzsche *et al.* (1990) *Science* 249:283-287.

Briefly, MHC-peptide complexes may be isolated by a conventional immunoaffinity procedure. Then the peptides are eluted from the MHC-peptide complex by incubating the complexes in the presence of about 0.1% TFA in acetonitrile. The extracted peptides may be fractionated and purified by reverse phase HPLC, as before.

The amino acid sequences of the eluted peptides may be determined either by manual or automated amino acid sequencing techniques well known in the art. Once the amino acid sequence of a potentially protective peptide has been determined the peptide may be synthesized in any desired amount using conventional peptide synthesis or other protocols well known in the art.

V. Synthesis of Potentially Useful Immunogenic Peptides.

Peptides having the same amino acid sequence as those isolated above may
5 be synthesized by solid-phase peptide synthesis using procedures similar to those
described by Merrifield (1963) J. Am. Chem. Soc., 85: 2149. During synthesis, N- α -
protected amino acids having protected side chains are added stepwise to a
growing polypeptide chain linked by its C-terminal end to an insoluble polymeric
support i.e., polystyrene beads. The peptides are synthesized by linking an amino
10 group of an N- α -deprotected amino acid to an α -carboxy group of an N- α -protected
amino acid that has been activated by reacting it with a reagent such as
dicyclohexylcarbodiimide. The attachment of a free amino group to the activated
carboxyl leads to peptide bond formation. The most commonly used N- α -
protecting groups include Boc which is acid labile and Fmoc which is base labile.

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Briefly, the C-terminal N- α -protected amino acid is first attached to the
polystyrene beads. The N- α -protecting group is then removed. The deprotected α -
amino group is coupled to the activated α -carboxylate group of the next N- α -
protected amino acid. The process is repeated until the desired peptide is
20 synthesized. The resulting peptides then are cleaved from the insoluble polymer
support and the amino acid side chains deprotected. Longer peptides can be
derived by condensation of protected peptide fragments. Details of appropriate
chemistries, resins, protecting groups, protected amino acids and reagents are well
known in the art and so are not discussed in detail herein. See for example,
25 Atherton et al., Solid Phase Peptide Synthesis: A Practical Approach, IRL Press,
(1989), and Bodanszky, Peptide Chemistry, A Practical Textbook, 2nd Ed, Springer-
Berlog (1993), the disclosures of which are incorporated herein by reference.

- 43 -

Purification of the resulting peptides is accomplished using conventional procedures, such as preparative HPLC using gel permeation, partition and/or ion exchange chromatography. The choice of appropriate matrices and buffers are well known in the art and so are not described in detail herein.

5

VI. Reconstitution of Stress Protein-peptide Complexes.

As will be appreciated by those skilled in the art, the peptides, either isolated from the complexes using the aforementioned procedures or chemically synthesized, may be reconstituted with a variety of naturally purified or recombinant stress proteins in vitro to generate immunogenic stress protein-peptide complexes. A preferred protocol for reconstituting a stress protein and a peptide in vitro is discussed below.

15 Prior to reconstitution the stress proteins are pretreated with ATP or low pH to remove any peptides that may be associated with the stress-protein of interest. When the ATP procedure is used, excess ATP is removed from the preparation by the addition of apyranase as discussed in Levy *et al.* (1991) *Cell* 67:265-274, the disclosure of which is incorporated herein by reference. When the low pH
20 procedure is used the buffer is readjusted to neutral pH by the addition of pH modifying reagents.

The peptide (1mg) and the pretreated stress protein (9mg) are admixed to give an approximate molar ratio of 5 peptides:1 stress protein. Then, the mixture is
25 incubated for 5 hours at room temperature in a binding buffer containing 20mM sodium phosphate, pH 7.2, 350mM NaCl, 3mM MgCl₂, 1mM PMSF. The preparations are centrifuged through Centricon 10 assembly (Millipore) to remove any unbound peptide. The association of the peptides with the stress proteins can

- 44 -

be assayed by SDS-PAGE and radioautography when radiolabelled peptides are used to reconstitute the complexes.

Following reconstitution, the candidate immunogenic stress protein-peptide
5 complexes can be tested in vitro using for example the mixed lymphocyte target cell assay (MLTC) described below. Once potential immunogenic constructs have been isolated they can be characterized further in animal models using the preferred administration protocols and excipients discussed below.

10 VII. Determination of Immunogenicity of Stress Protein-Peptide Complexes.

The purified and reconstituted stress protein-peptide complexes can be assayed for immunogenicity using the mixed lymphocyte target-culture assay (MLTC) well known in the art.

15

Briefly, mice are injected subcutaneously with the candidate stress protein-peptide complexes. Other mice are injected with either other stress protein-peptide complexes or whole infected cells which act as positive controls for the assay. The mice are injected twice, 7-10 days apart. Ten days after the last immunization, the
20 spleens are removed and lymphocytes released from the excised spleens. The released lymphocytes may be restimulated in vitro by the subsequent addition of dead cells which prior to death had expressed the complex of interest.

For example, 8×10^6 immune spleen cells may be stimulated with either
25 4×10^4 mitomycin C treated or γ -irradiated (5-10,000 rads) cells (the cells having been infected with the intracellular pathogen or transfected with an appropriate gene) in 3ml RPMI medium containing 10% fetal calf serum. In certain cases 33% secondary mixed lymphocyte culture supernatant may be included in the culture medium as a source of T cell growth factors. See for example, Glasebrook et al.

- 45 -

(1980) J. Exp. Med. 151:876. In order to test the primary cytotoxic T cell response after immunization, spleen cells may be cultured without stimulation. In some experiments spleen cells of the immunized mice also may be restimulated with antigenically distinct cells, to determine the specificity of the cytotoxic T cell response.

Six days later the cultures are tested for cytotoxicity in a 4 hour ^{51}Cr -release assay. See for example, Palladino et al. (1987) Cancer Res. 47:5074-5079 and Blachere et al. (1993) J. Immunotherapy 14:352-356, the disclosures of which are incorporated herein by reference. In this assay, the mixed lymphocyte culture is added to a target cell suspension to give different effector:target (E:T) ratios (usually 1:1 to 40:1). The target cells are prelabelled by incubating 1×10^6 target cells in culture medium containing 200 mCi ^{51}Cr /ml for one hour at 37°C . The cells are washed three times following labeling. Each assay point (E:T ratio) is performed in triplicate and the appropriate controls incorporated to measure spontaneous ^{51}Cr release (no lymphocytes added to assay) and 100% release (cells lysed with detergent). After incubating the cell mixtures for 4 hours, the cells are pelleted by centrifugation at 200g for 5 minutes. The amount of ^{51}Cr released into the supernatant is measured by a gamma counter. The percent-cytotoxicity is measured as cpm in the test sample minus spontaneously released cpm divided by the total detergent released cpm minus spontaneously released cpm.

In order to block the MHC class I cascade a concentrated hybridoma supernatant derived from K-44 hybridoma cells (an anti-MHC class I hybridoma) is added to the test samples to a final concentration of 12.5%.

- 46 -

VIII. Formulation and Vaccination Regimes.

Once candidate stress protein-peptide complexes have been identified they
5 may be administered either to an animal model or to the intended recipient to
stimulate cytotoxic T cell responses against the preselected intracellular pathogen.
The stress protein-peptide complexes of the invention may be either stored or
prepared for administration by mixing with physiologically acceptable carriers,
excipients, or stabilizers. These materials should be non-toxic to the intended
10 recipient at dosages and concentrations employed.

If the complex is water soluble then it may be formulated in an appropriate
buffer, for example phosphate buffered saline (5mM sodium phosphate, 150 mM
NaCl, pH7.1) or other physiologically compatible solutions. Alternatively, if the
15 resulting complex has poor solubility in aqueous solvents then it may be
formulated with a non-ionic surfactant such as Tween, or polyethylene glycol.

Useful solutions for oral or parenteral administration may be prepared by
any of the methods well known in the pharmaceutical art, described, for example,
20 in Remington's Pharmaceutical Sciences, (Gennaro, A., ed.), Mack Pub., 1990.
Formulations may include, for example, polyalkylene glycols such as polyethylene
glycol, oils of vegetable origin, hydrogenated naphthalenes, and the like.
Formulations for direct administration, in particular, may include glycerol and
other compositions of high viscosity. Biocompatible, preferably bioresorbable
25 polymers, including, for example, hyaluronic acid, collagen, tricalcium phosphate,
polybutyrate, polylactide, polyglycolide and lactide/glycolide copolymers, may be
useful excipients to control the release of the stress protein-peptide complexes in
vivo.

- 47 -

Formulations for inhalation administration may contain as excipients, for example, lactose. Aqueous solutions may contain, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate. Oily solutions may be useful administration in the form of nasal drops. Gels may be applied topically
5 intranasally.

The compounds provided herein can be formulated into pharmaceutical compositions by admixture with pharmaceutically acceptable non toxic excipients and carriers. In addition the formulations may optionally contain one or more
10 adjuvants. Preferred adjuvants include, but are not limited to, pluronic tri-block copolymers, muramyl dipeptide and its derivatives, detoxified endotoxin, saponin and its derivatives such as QS-21 and liposomes. The present invention further envisages sustained release formulations in which the complex is released over an extended period of time.

15 The dosage and means of administration of the family of stress protein-peptide vaccines prepared in accordance with the invention will necessarily depend upon the nature of the complex, the intracellular pathogen and the nature of the disease in question. The complex should be administered in an amount sufficient
20 to initiate a cytotoxic T cell response against the intracellular pathogen. The preferred dosage of drug to be administered also is likely to depend on such variables as the type of disease, the age, sex and weight of the intended recipient, the overall health status of the particular patient, the relative biological efficacy of the compound selected, the formulation of the compound, the presence and types
25 of excipients in the formulation, and the route of administration.

In general terms, the compounds of this invention may be provided in an aqueous physiological buffer solution containing about 0.001 to 10% w/v compound for parenteral administration. Typical doses range from about 0.1 to

- 48 -

about 1000 micrograms of complex/kg body weight of recipient/immunization; and preferably range from about 0.5 to about 100 micrograms of complex/kg body weight of recipient/immunization. It is contemplated that between about 10 to about 250 micrograms of complex will be administered per dose to a human subject
5 weighing about 75kg. These quantities, however, may vary according to the adjuvant-co-administered with the complex.

The vaccines may be administered using standard protocols which include, but are not limited to, intramuscular, subcutaneous, intradermal, intraperitoneal,
10 intravenous, intravaginal, intrarectal, oral, sublingual, transcutaneous, and intranasal administration. Preferably the recipient should be vaccinated four times at weekly intervals. If necessary, the responses may be boosted at a later date by subsequent administration of the vaccine. It is contemplated that the optimal dosage and vaccination schedule may be determined empirically for each stress
15 protein-peptide vaccine using techniques well known in the art.

Various cytokines, antibiotics, and other bioactive agents also may be administered with the stress protein complexes. For example, various known cytokines, i.e., IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11,
20 IL-12, IFN α , IFN β , IFN γ , TNF α , TNF β , G-CSF, GM-CSF, and TGF- β may be co-administered with the complexes in order to maximize the physiological response. It is anticipated, however, that other but as yet undiscovered cytokines may be effective in the invention. In addition, conventional antibiotics may be co-administered with the stress protein-peptide complex. The choice of suitable
25 antibiotics will also depend upon the disease in question.

- 49 -

Example 1. Immunogenicity of Stress Protein-peptide Complexes Isolated from Cells Transfected with a Gene Encoding an Antigenic Determinant.

5 Figure 1 shows the antigen specific cytotoxic T cell activity of splenocytes derived from mice immunized with a gp96-peptide complex harvested from BALB/c fibroblasts transfected with the nucleoprotein (NP) gene from the PR8 influenza virus.

10 Briefly, gp96-peptide preparations were isolated from BALB/c cells transfected with and expressing the NP gene of the PR8 influenza virus. The gp96-peptide complex was isolated from 100,000g supernatant by the unmodified gp60-peptide complex purification protocol. Then, the preparations were used to immunize naive BALB/c mice. The mice were injected twice subcutaneously with
15 the gp96-peptide complexes at ten day intervals. The mice were sacrificed and the spleen cells obtained. The spleen cells were stimulated twice *in vitro* by the additional lethally irradiated BALB/c cells expressing the NP gene using the mixed target lymphocyte culture (MLTC) assay described above. Six days later the
20 cultures were tested for cytotoxicity using the ^{51}Cr release assay. In order to block the MHC type I cascade the spleen cells were incubated with the supernatant derived from K-44 hybridoma (containing anti-MHC type I immunoglobulins) culture.

 The cytotoxic activity was assayed by the release of ^{51}Cr from BALB/c
25 fibroblasts expressing the NP gene (filled circles), BALB/c cells expressing the NP gene but treated with the anti-MHC type I antisera (empty circles) and from the syngeneic non-NP transfected cell-line 5117 (asterisks). The spleens of the mice immunized with the gp96 complex showed strong MHC class I-restricted cytotoxic T cell activity against BALB/c cells expressing the NP gene, but not against the

- 50 -

syngeneic non-NP transfected cell line 5117. Furthermore, the anti MHC type I antisera blocked the response. Therefore, it is apparent that immunization with a stress protein-peptide complex elicits a specific cytotoxic T cell response against the peptide in the complex and that the MHC class I cascade plays an integral role in stimulating the cytotoxic T cell response against cells infected with intracellular pathogens.

Example 2. Immunogenicity of Stress Protein-peptide Complexes Isolated from SV40 Transformed Cells.

10

Figure 2 shows the antigen specific cytotoxic T cell activity of splenocytes derived from mice immunized with gp96-peptide complex harvested from SV40 transformed SVB6 cells.

15

Briefly, gp96-peptide complexes were isolated from SV40 transformed SVB6 cells and used to immunize naive (57BL/6) mice. The gp96-peptide complex was isolated from 100,000g supernatant by the unmodified gp60-peptide complex purification protocol. The mice were injected twice subcutaneously with the complex at ten day intervals. The mice were sacrificed, the spleen cells isolated and stimulated in vitro by the addition of lethally irradiated SV40 transformed SVB6 cells by the MLTC procedure. Six days later the cells were assayed for cytotoxicity using the ^{51}Cr release assay. The cytotoxic activity was assayed by the release of ^{51}Cr from SVB6 cells (filled triangles) and from a non SV40 transfected syngeneic cell line, MCA (empty triangles). MHC class I mediated activity was assayed also by adding anti-MHC class I immunoglobulins derived from the K-44 hybridoma cell line to the spleen cells.

20

25

- 51 -

The spleen cells isolated from mice immunized with the gp96-peptide complex showed strong MHC class I-restricted activity against the SV40 transfected SVB6 cells but not against the non transfected cells.

5

Example 3. Reconstitution of Immunogenic Stress
Protein-peptide Complexes In Vitro.

Figures 3A-3D show antigen specific cytotoxic T cell activities of splenocytes
10 derived from two mice immunized with reconstituted Hsp70-peptide complex.

Briefly, uncomplexed Hsp70 was purified by the procedure described above and the peptide (SLSDLRGYVYQGL, SEQ. ID. NO.: 1) was synthesized by solid phase peptide synthesis. The peptide (1mg) and ATP treated Hsp70 (9mg) were
15 admixed and incubated for 3 hours at room temperature in a binding buffer containing 20mM sodium phosphate, pH 7.2, 350mM NaCl, 3mM MgCl₂, 1mM PMSF. The resulting preparation was centrifuged through Centricon 10 assembly (Millipore) to remove unbound peptide.

20 The resulting complex was used to immunize two naive mice. The spleen cells were isolated from the mice and stimulated twice in vitro by the addition of lethally irradiated EL4 cells transfected with, and expressing a minigene encoding the peptide SLSDLRGYVYQGL (SEQ. ID. NO.: 1), using the MLTC procedure. The cytotoxicities of spleen cells from both mice were assayed after the first (3A and 3C)
25 and second (3B and 3D) stimulations by the ⁵¹Cr release assay. The release of ⁵¹Cr was measured from EL4 cells (hollow triangles) and from EL4 cells transfected with, and expressing the peptide SLSDLRGYVYQGL (SEQ. ID. NO.: 1) (filled triangles). The results show that stress proteins and peptides can be reconstituted

- 52 -

successfully in vitro to give specific immunogenic stress protein-peptide complexes.

The invention may be embodied in other specific forms without departing
5 from the spirit or essential characteristics thereof. The present embodiments are
therefore to be considered in all respects as illustrative and not restrictive, the scope
of the invention being indicated by the appended claims rather than by the
foregoing description, and all changes which come within the meaning and range
of equivalency of the claims are therefore intended to be embraced therein.

10

- 53 -

SEQUENCE LISTING

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15 (I) TELEX:

(ii) TITLE OF INVENTION: STRESS PROTEIN-PEPTIDE COMPLEXES AS
PROPHYLACTIC AND THERAPEUTIC VACCINES AGAINST
INTRACELLULAR PATHOGENS

20

(iii) NUMBER OF SEQUENCES: 1

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(v) COMPUTER READABLE FORM:

- 54 -

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

5

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(viii) ATTORNEY / AGENT INFORMATION:

- (A) NAME: PITCHER, EDMUND R
- (B) REGISTRATION NUMBER: 27,829
- (C) REFERENCE / DOCKET NUMBER: ARM-001

15

(ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: (617) 248-7000
- (B) TELEFAX: (617) 248-7100

20 (2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: peptide

30

(ix) FEATURE:

- (A) NAME / KEY: Peptide
- (B) LOCATION: 1..13

- 55 -

(D) OTHER INFORMATION: /label= PEPTIDE1

/note= "ANTIGENIC PEPTIDE I"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

5

Ser Leu Ser Asp Leu Arg Gly Tyr Val Tyr Gln Gly Leu

1

5

10

- 56 -

What is claimed is:

1. A vaccine for administration to a mammal for inducing in the mammal a cytotoxic T cell response against a preselected intracellular pathogen, the vaccine
5 comprising:
 - (a) an immunogenic stress protein-peptide complex operative to initiate in said mammal a cytotoxic T cell response against said pathogen, said complex comprising,
10 a peptide that is present in a eukaryotic cell infected with said pathogen but not present in said cell when said cell is not infected with said pathogen, complexed with a stress protein; and
 - (b) a pharmaceutically acceptable carrier.
2. A vaccine for administration to a mammal for inducing in said mammal
15 resistance to infection by a preselected intracellular pathogen, the vaccine comprising:
 - (a) an immunogenic stress protein-peptide complex operative to initiate in said mammal, by means of a cytotoxic T cell response in said mammal, resistance to infection by said pathogen, said complex comprising,
20 a peptide that is present in a eukaryotic cell infected with said pathogen but not present in said cell when said cell is not infected with said pathogen, complexed with a stress protein; and
 - (b) a pharmaceutically acceptable carrier.
- 25 3. The composition of claim 1 or 2, wherein said stress protein is a member of the stress protein families selected from the group consisting of Hsp60, Hsp70, and Hsp90.
4. The composition of claim 1 or 2, wherein said stress protein is a gp96.

- 57 -

5. The composition of claim 1 or 2 further comprising a cytokine.
6. The composition of claim 5, wherein said cytokine is selected from the
5 group consisting of IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IFN α , IFN β , IFN γ , TNF α , TNF β , G-CSF, GM-CSF, GM-CSF and TGF- β .
7. The composition of claim 1 or 2, wherein said eukaryotic cell is an
immortalized eukaryotic cell.
10
8. The composition of claim 1 or 2, wherein said mammal is a human.
9. The composition of claim 1 or 2, wherein said pathogen is a virus.
- 15 10. The composition of claim 9, wherein said virus is selected from the group
consisting of hepatitis type A, hepatitis type B, hepatitis type C, influenza, varicella,
adenovirus, herpes simplex type I, herpes simplex type II, rinderpest, rhinovirus,
echovirus, rotavirus, respiratory syncytial virus, papilloma virus, papova virus,
cytomegalovirus, echinovirus, arbovirus, huntavirus, coxsachie virus, mumps
20 virus, measles virus, rubella virus, polio virus, human immunodeficiency virus
type I, and human immunodeficiency virus type II.
11. The composition of claim 1 or 2, wherein said pathogen is a bacteria.
- 25 12. The composition of claim 11, wherein said bacteria is selected from the
group consisting of *Mycobacteria*, *Rickettsia*, *Neisseria* and *Legionella*.
13. The composition of claim 1 or 2, wherein said pathogen is a protozoa.

- 58 -

14. The composition of claim 13, wherein said protozoa is selected from the group consisting of *Leishmania*, *Trypanosoma* and *Kokzidia*.
15. The composition of claim 1 or 2, wherein said pathogen is an intracellular parasite.
16. The composition of claim 15, wherein said parasite is selected from the group consisting of *Chlamydia* and *Rickettsia*.
17. The composition of claim 1 or 2, wherein said peptide is non covalently complexed with said stress protein.
18. A method of inducing in a mammal a cytotoxic T cell response against a preselected intracellular pathogen that causes disease in said mammal, the method comprising:
- administering to said mammal a vaccine comprising,
 - (a) an immunogenic stress protein-peptide complex operative to initiate in said mammal a cytotoxic T cell response against said pathogen and comprising,
 - a peptide that is present in a eukaryotic cell infected with said pathogen but not present in said cell when said cell is not infected with said pathogen, complexed with a stress protein, and
 - (b) a pharmaceutically acceptable carrier,
- in an amount sufficient to elicit in said mammal a cytotoxic T cell response against said pathogen.
19. A method of inducing in a mammal resistance to infection by a preselected intracellular pathogen that-causes disease in said mammal, the method comprising:

- 59 -

administering to said mammal a vaccine comprising,

- 5 (a) an immunogenic stress protein-peptide complex
operative to initiate in said mammal cytotoxic T cell
response against said pathogen and comprising,
a peptide that is present in a eukaryotic cell
infected with said pathogen but not present in
said cell when said cell is not infected with
said pathogen, complexed with a stress
protein, and
- 10 (b) a pharmaceutically acceptable carrier,
in an amount sufficient to induce in said mammal, by means of the
cytotoxic T cell response in said mammal, resistance to infection by
said pathogen.
- 15 20. The method of claim 18 or 19, wherein said cytotoxic T cell response is
mediated by the class I major histocompatibility complex.
21. The method of claim 18 or 19, wherein said stress protein is a member of the
stress protein families selected from the group consisting of Hsp60, Hsp70, and
20 Hsp90.
22. The method of claim 18 or 19, wherein said stress protein is a gp96.
23. The method of claim 18 or 19, wherein said composition further comprises a
25 cytokine.
24. The method of claim 23, wherein said cytokine is selected from the group
consisting of IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-
12, IFN α , IFN β , IFN γ , TNF α , TNF β , G-CSF, GM-CSF, GM-CSF and TGF- β .

- 60 -

25. The method of claim 18 or 19, wherein said eukaryotic cell is an immortalized eukaryotic cell.
- 5 26. The method of claim 18 or 19, wherein said mammal is a human.
27. The method of claim 18 or 19, wherein said vaccine is administered prophylactically to said mammal for stimulating in said mammal a cytotoxic T cell response for preventing subsequent infection of said mammal by said pathogen.
- 10 28. The method of claim 18 or 19, wherein said vaccine is administered therapeutically to said mammal for stimulating in said mammal a cytotoxic T cell response against said pathogen presently infecting said mammal.
- 15 29. The method of claim 18 or 19, wherein said vaccine is administered to said mammal in an amount in the range of about 0.1 to about 1000 micrograms of complex/kg body weight of mammal/immunization.
- 20 30. The method of claim 29, wherein said amount is in range of about 0.5 to about 100 micrograms of complex/kg body weight of mammal/immunization.
31. A method for preparing a vaccine for inducing in a mammal a cytotoxic T cell response against a preselected intracellular pathogen, the method comprising:
- (a) harvesting from a eukaryotic cell infected with said pathogen
- 25 an immunogenic stress protein-peptide complex comprising,
- a peptide that is present in said cell infected with said pathogen but not present in said cell when said cell is not infected with said pathogen complexed with a stress protein, said complex, when administered to said mammal, being

- 61 -

operative at initiating in said mammal a cytotoxic T cell response against said pathogen; and

(b) combining said complex with a pharmaceutically acceptable carrier.

5 32. A method for preparing a vaccine for inducing in a mammal a cytotoxic T cell response against a preselected intracellular pathogen, the method comprising:

(a) reconstituting in vitro,

10 a peptide that is present in a eukaryotic cell infected with said pathogen but not present in said cell when said cell is not infected with said pathogen and a stress protein, thereby to generate a stress protein-peptide complex, which when administered to said mammal is operative to initiate a cytotoxic T cell response against said pathogen in said mammal; and

(b) combining said complex with a pharmaceutically acceptable carrier.

15

33. The method of claim 32, wherein said stress protein is harvested in the presence of ATP prior to reconstitution.

20 34. The method of claim 32, wherein said stress protein is treated with low pH prior to reconstitution.

35. The method of claim 31 or 32, wherein said stress protein is a member of the stress protein families selected from the group consisting of Hsp60, Hsp70 and
25 Hsp90.

36. The method of claim 31 or 32, wherein said stress protein is a gp96.

1/3

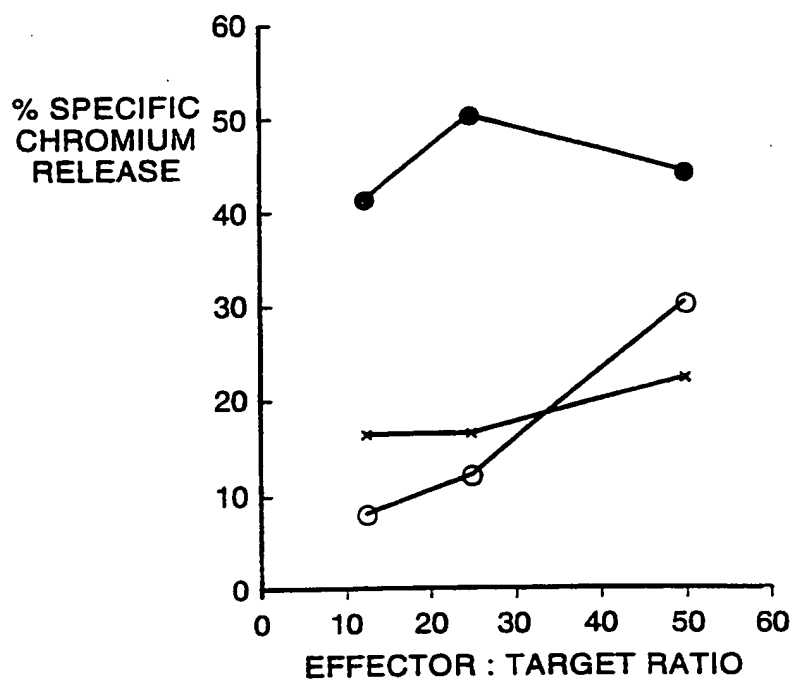


FIG. 1

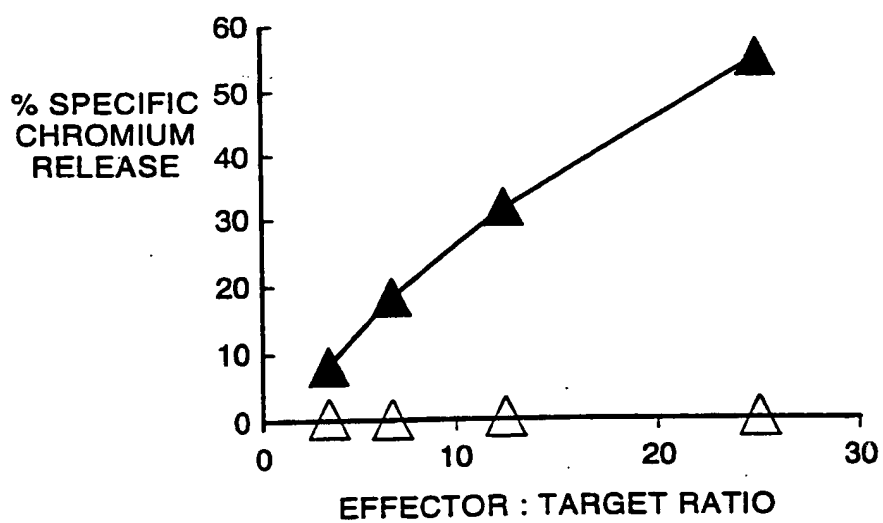


FIG. 2

2/3

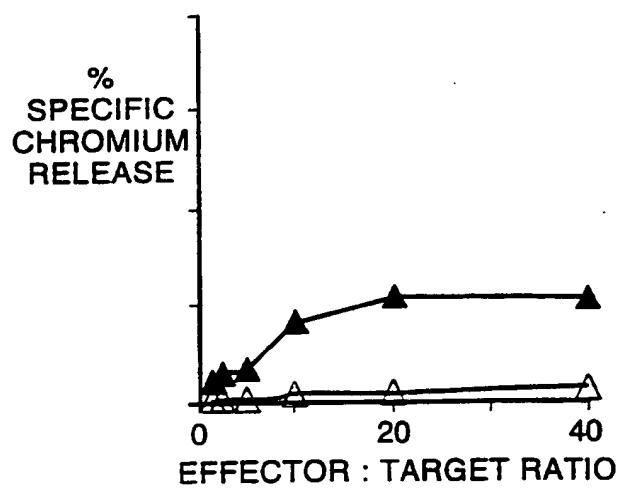


FIG. 3A

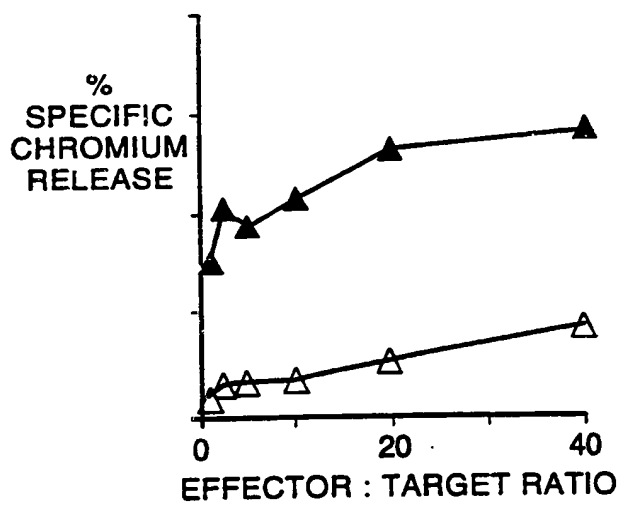


FIG. 3B

3/3

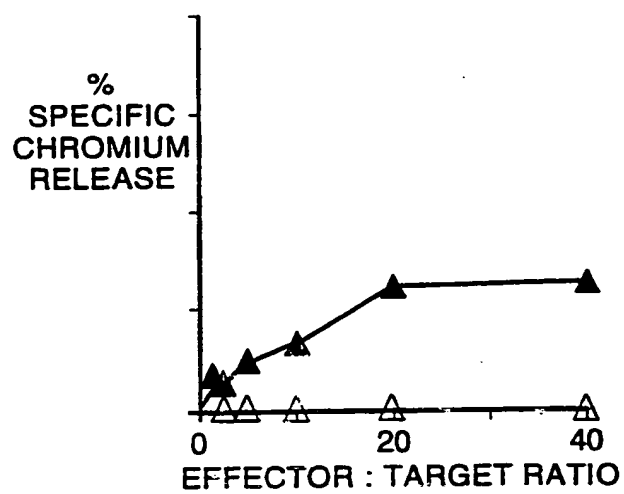


FIG. 3C

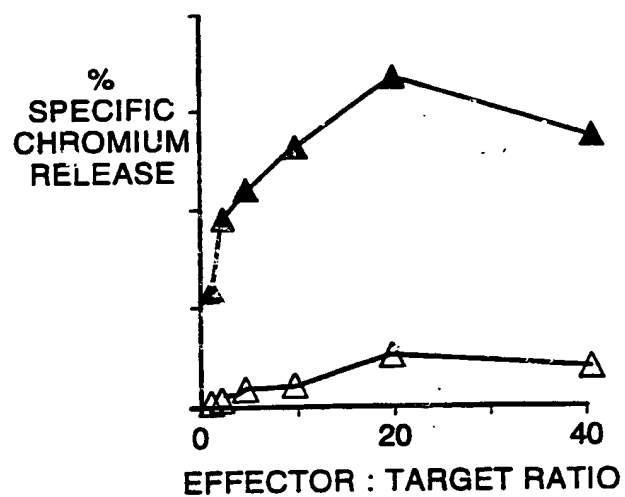


FIG. 3D

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(54) Title: STRESS PROTEIN-PEPTIDE COMPLEXES AS PROPHYLACTIC AND THERAPEUTIC VACCINES AGAINST INTRA-CELLULAR PATHOGENS (57) Abstract Disclosed is a family of vaccines that contain stress protein-peptide complexes which when administered to a mammal are operative to initiate in the mammal a cytotoxic T cell response against cells infected with a preselected intracellular pathogen. Also disclosed are methodologies for preparing and administering vaccines containing such stress protein-peptide complexes.		

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CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
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FR	France			VN	Viet Nam
GA	Gabon				

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 95/03311

A. CLASSIFICATION OF SUBJECT MATTER IPC 6 A61K39/002 A61K39/02 A61K39/118 A61K39/12 A61K39/295 C07K17/00		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 6 A61K C07K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EUR. J. IMMUNOL., vol. 22, 1992 pages 1365-1372, C. BARRIOS ET AL. 'Mycobacterial heat-shock proteins as carrier molecules. II: The use of the 70-kDa mycobacterial heat-shock protein as carrier for conjugated vaccines can circumvent the need for adjuvants and Bacillus Calmette Guérin priming.' see page 1365, paragraph 1 - page 1366 see page 1366, paragraph 2.2 see page 1370, paragraph 4	1-3, 11, 12, 17
Y	Discussion, first and second paragraphs --- -/--	4-36
<div style="display: flex; justify-content: space-between;"> <div> <input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. </div> <div> <input checked="" type="checkbox"/> Patent family members are listed in annex. </div> </div>		
<div style="display: flex;"> <div style="flex: 1;"> <p>* Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="flex: 1;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>		
Date of the actual completion of the international search <div style="text-align: center;">14 July 1995</div>		Date of mailing of the international search report <div style="text-align: center;">2 9. 08. 95</div>
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016		Authorized officer <div style="text-align: center;">Halle, F</div>

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 95/03311

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	CHEMICAL ABSTRACTS, vol. 121, no. 23, 5 December 1994 Columbus, Ohio, US; abstract no. 271089a, SRIVASTAVA P.K. AND UDONO H. 'Heat-shock protein-peptide complexes in cancer immunotherapy.' page 12;	1-3
Y	see abstract & CURR. OPIN. IMMUNOL., vol. 6, no. 5, 1994 pages 728-732, ---	4-36
X,P	WO-A-94 29459 (WHITEHEAD BIOMEDICAL INST) 22 December 1994 see the whole document ---	1-3
Y	WO-A-94 03208 (YEDA RES & DEV ;COHEN IRUN R (IL); FRIDKIN MATITYAHU (IL); KONEN W) 17 February 1994 see page 7, line 17 - page 8, line 32 see claims 1-5,20-25 ---	4-36
A	WO-A-93 18146 (INST NAT SANTE RECH MED) 16 September 1993 see the whole document ---	1-3
A	EXPERIENTIA, vol. 50, 1994 pages 1054-1060, P. K. SRIVASTAVA 'Heat shock proteins in immune response to cancer: The Fourth Paradigm.' -----	

INTERNATIONAL SEARCH REPORT

national application No.

PCT/US95/03311

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 18-30
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 18-30 are directed to a method of treatment of the human or animal body the search has been carried out and based on the alleged effects of the composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/US 95/03311

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9429459	22-12-94	NONE	
<hr/>			
WO-A-9403208	17-02-94	AU-B- 4790093	03-03-94
		CA-A- 2141454	17-02-94
		EP-A- 0658120	21-06-95
		FI-A- 950405	30-03-95
		NO-A- 950328	23-03-95
<hr/>			
WO-A-9318146	16-09-93	FR-A- 2688227	10-09-93
<hr/>			

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